VITAMIN A METABOLISM IN RETINAL FUNCTION

F. LION

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Aan Shilly

en aan mijn ouders

CONTENTS

The importance of vitamin A in the function of the photosensitive receptor cells of the vertebrate retina has long ago been established by Wald (1933), but many problems concerning its metabolic events in the retina, especially its isomerisation in the rhodopsin regeneration process, remain to be solved.

After two introductory chapters about the anatomy and physiology of the vertebrate retinal photoreceptors and a chapter concerned with the clinical and experimental retinal photoreceptor degenerations, the subsequent chapters deal with our experiments on various aspects of vitamin A metabolism in the vertebrate retina.

We have first tried to investigate the conditions under which rhodopsin would regenerate in an isolated rat retina system (chapter 4). Thereafter, we have investigated the properties of retinoldehydrogenase in cattle retina preparations, in particular its stereospecificity towards vitamin A compounds, in order to reach a better understanding about the possible pathways of these compounds in the retina during light and dark adaptation (chapter 5). Because of the established, but in no way completely understood role of the pigment epithelium in the vitamin A metabolism, we have used the same approach for the enzyme in cattle pigment epithelium (chapter 6). For a better functional understanding of the retinoldehydrogenases described in chapter 5 and 6 we have studied the localization of these enzymes in the photoreceptors and the pigment epithelial cells by subcellular fractionation methods (chapter 7).

In the last experimental chapter (chapter 8) various investigations are described concerning retinal metabolism in experimental animals known to have an inherited retinal dystrophy.

CHAPTER 1

DEVELOPMENTAL ANATOMY OF THE VERTEBRATE RETINA

1.1 Embryology of the eye and histogenesis of the retina

The eye is derived from ectodermal, neural ectodermal and mesodermal tissue. The neural ectodermal part develops as a lateral diverticulum of the prosencephalon, the foremost part of the developing brain. This diverticulum is called the optic vesicle. At first each optic vesicle possesses a cavity continuous with that of the prosencephalon. The proximal portion of the vesicle becomes relatively constricted to form the optic stalk, which upon maturation forms the optic nerve. The distal part of the optic vesicle is invaginated into its more proximal part, so that it is converted into a double-layered optic cup. (See e.g. Rodieck 1973 pp 338-429).

The cells of the thinner outer layer of the optic cup early acquire pigment and develop into the pigment epithelium of the retina. The inner layer of the optic cup differentiates into the sensory retina. The zone of the retina in contact with the pigment epithelium differentiates into the rod and cone cells. So both the pigment epithelium and the photoreceptor layer are derived from the same lining epithelium of the embryonic forebrain. The choroid is derived from mesodermal tissue surrounding the optic vesicle.

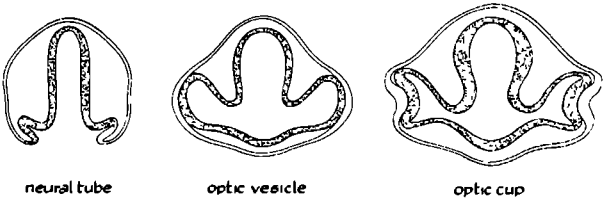

Fig. 1 Schematic diagram of the development of the vertebrate eye.

1.2 Anatomy of the vertebrate retina

The mature vertebrate retina consists of the pigment epithelium and the pars optica retinae. The pars optica retinae is formed by three layers of nerve cells and their synapses: the photoreceptor layer, a relay layer of bipolar cells and horizontal cells, and a layer of ganglion cells, the axons of which run via the optic nerve into the central nervous system. These cell layers are bound together by neuroglia cells, the Müller cells. The structure of the vertebrate retina is depicted in fig. 2. With the light microscope one can identifie ten layers in the mature retina: 1. the retinal pigment epithelium; 2. the rod and cone layer; 3. the outer limiting membrane, produced by terminal bars uniting the cell membranes of the rods and cones to the Müller cells; 4. the outer nuclear layer formed by the nuclei of rod and cone cells; 5. the outer plexiform layer, the site of the synapses between the photoreceptor cells and the bipolar and horizontal cells; 6. the inner nuclear layer containing the cell nuclei of horizontal cells, Müller cells, bipolar cells and amacrine cells; 7. the inner plexiform layer formed by the processes of the bipolar cells, amacrine cells and ganglion cells; 8. the ganglion cell layer; 9. a nerve fiber layer; 10. the inner limiting membrane, formed by processes of the Müller cells, which separates the sensory retina from the vitreous.

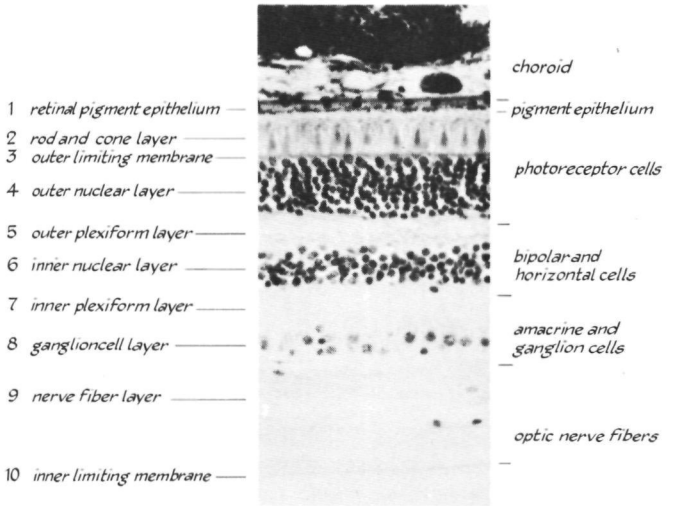

Fig. 2 Light microscopic structure of the vertebrate retina.

Visual excitation takes place in the photoreceptor layer. There are, however, close metabolic relations between pigment epithelial cells, photoreceptor cells and Müller cells, so in addition to the photoreceptor cells, the other two types of cells will be discussed in some detail. Since in man and other vertebrates the rods, which are the dim light receptors, are much more numerous than the cones, biochemical research has been largely confined to the rods. Hence, the cones will not be discussed here.

1.2.1 Anatomy of the retinal pigment epithelium

The retinal pigment epithelium is a single layer of uniform hexagonal cells adjacent to the sensory retina, extending from the edge of the optic disc to the ora serrata. The pigment epithelium is continuous with the outer ciliary epithelium.

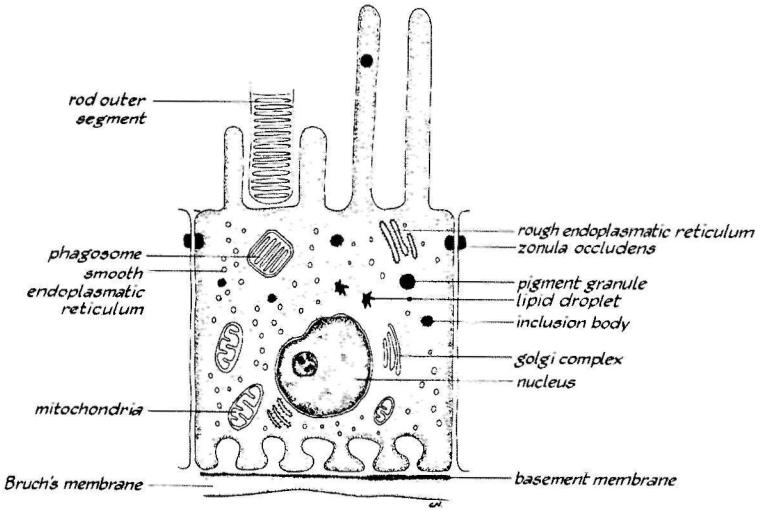

Fig. 3 Schematic diagram of a pigment epithelial cell

The cell membrane at the base of the cell facing Bruch's membrane shows marked complex infoldings into the cytoplasm. Between the cell membrane and Bruch's membrane lies a basement membrane which does not follow the cell membrane infoldings. The lateral cell membranes are only slightly interdigitated. The intercellular spaces are sealed off from the apical lumen by zonulae occludentes. The apical cell membrane, facing the rod

and cone receptors shows many microvilli which are of two types, long slender villi that stretch between the outer segments of the photoreceptors, and short broad villi that form a sheath around the terminations of the outer segments. The microvilli surround the external third of the outer processes of the rods and cones. A viscous ground substance rich in mucopolysaccharides surrounds the villi as well as the outer and inner segments of the rods and cones.

The cytoplasm of the pigment epithelial cells contains a rather large nucleus located in the basal portion of the cell. Many large pigment granules are present in the cytoplasm, mainly in the upper and middle part of the cell. The cytoplasmic structure, when viewed by electron microscopy, is dense and complex. It contains many free ribosomes and numerous small mitochondria. Smooth surfaced endoplasmic reticulum occupies the greater portion of the cytoplasm. The rough surfaced endoplasmic reticulum is poorly developed in the major part of the pigment epithelium. It is found in the apical portion of the cells. Inclusion bodies of various kinds which are found commonly in the cytoplasm are believed to function in degrading phagocytized substances. Newly engulfed pieces of rod outer segments are often present. The cytoplasm also contains lipofuscin granules, which probably represent the final product of phagocytic activity (Moyer, 1969; Hogan, Alvarado and Weddell, 1971).

1.2.2 Anatomy of the rod photoreceptor cell

The rods and cones are located externally to the outer limiting membrane. The rods are composed of a relatively broad inner segment and a narrow outer segment, joined by a constriction, the connecting cilium (fig. 4). The rod outer segments are composed of a pile of flat sacs or discs, surrounded by a cell membrane. Each disc is formed by a unit membrane. The main protein constituent of these disc membranes is the visual pigment rhodopsin. The cell membrane of the rod surrounds the pile of discs and is continuous with that of the connecting cilium and the inner segment. The distal third part of the rod outer segment is surrounded by the short processes of the pigment epithelial cells. The proximal third is enveloped by extensions of the inner segment membranes. Only the central portions of the rod outer segments are not wrapped in

cellular extensions.

The connecting cilium originates in the basal body, which is located in the cytoplasm of the ellipsoid, the upper part of the inner segment. The cilium contains nine doublets of tubules arranged in a ring. It provides the only connection between the outer and inner segment of the rod.

The inner segments are elongated barrel-shaped structures containing a finely granular cytoplasm. They are subdivided into two parts, an ellipsoid that is adjacent to the outer segment, and a myoid, which is continuous with the soma of these cells in the outer nuclear layer. The rod ellipsoid contains a large number of mitochondria, glycogen granules, vesicles of smooth surfaced endoplasmic reticulum, neurotubules, many neurofibrils and free ribosomes. The myoid contains a considerable amount of the randomly oriented, smooth surfaced endoplasmic reticulum, which is arranged in round or oval vesicles. Many vesicles of the Golgi apparatus are usually seen close to the outer limiting membrane.

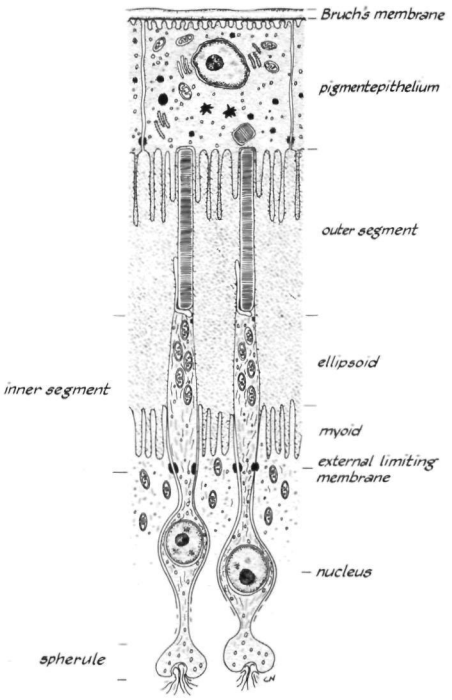

Fig. 4 Schematic diagram of a rod photoreceptor cell

Some rough surfaced endoplasmic reticulum is also present. Free ribosomes, which often form polyribosomes, and a small number of mitochondria are also present here. The inner segments are not in direct contact with adjacent receptor cells, but are separated from each other by thin villous extensions of Müller cells beyond the external limiting membrane. The nuclei are located in the inner segment at the level of the outer nuclear layer. Many neurotubules are aggregated around the nucleus and extend into the axon. The rod axon terminates in an oval structure located at the level of the plexiform layer, the spherule, which contains large numbers of presynaptic vesicles, mitochondria and some neurotubules and ribosomes. A synaptic cleft separates the cell membranes of the spherule and the enclosed processes of the bipolar and horizontal cells (Hogan et al., 1971; Young, R.W., 1969).

1.2.3 Anatomy of the Müller cell

Müller cells are giant cells which occupy the full thickness of the retina from the internal to the external limiting membrane and even extend beyond the external limiting membrane by lamellar expansions that embrace portions of the inner segments of the photoreceptors. Extensions of the Müller cells enclose most of the neurons, insulating the nerve fibers. In the retinal layers that contain blood vessels, Müller cell processes surround large areas of the capillary walls. The cytoplasm contains numerous well developed fibrils and smooth surfaced endoplasmic reticulum in about the same amount as in the cytoplasm of the pigment epithelium.

Müller cells are, apart from their supporting function, important for the energy metabolism of the retinal cells. In avascular retinas the Müller cells contain a high amount of glycogen, the polymerized storage form of glucose, which is the main substrate for energy metabolism of neural tissue (Young, 1969). In vascular retinas, e.g. human retinas, close contact between Müller cell processes and capillaries guarantees an easy exchange of metabolites of retinal energy metabolism between Müller cells and the blood. The presence of a considerable amount of lactic acid dehydrogenase activity and of an ability to synthesize and store glycogen, makes it probable that the Müller cell makes available glucose to other retinal cells (Cogan and Kuwabara, 1967).

PHYSIOLOGICAL ASPECTS OF VISION

2.1 Introduction

The photoreceptor layer of the human retina, where visual excitation takes place, contains rods and cones. The distribution of the rods and cones in the retina is depicted in fig. 5 (Østerberg, 1935).

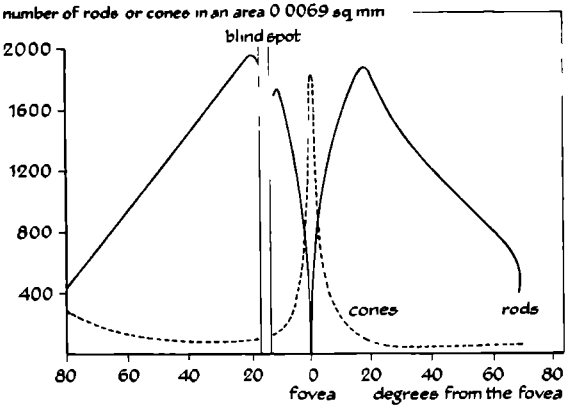

Fig. 5 The distribution of rods and cones in the retina

From this figure it is evident that the rods greatly outnumber the cones. Only in the macular area the concentration of cones is high. In the periphery the number of cones is small. The cones function at normal illumination levels and mediate color sensations. Cone malfunctioning, as in hereditary cone dystrophies and congenital color blindness, must have its basis in still unknown biochemical abnormalities resulting in cone degeneration. Unfortunately, little is known about the normal metabolism of the cones, since they cannot be isolated in bulk. The rods are present in large quantities in the retina, reaching a maximal concentration 15-20^{o} peripheral of the macula. Hence, they can be isolated relatively easily and thus they have been studied extensively biochemically. The rods function at low illumination intensities and discriminate only light intensity differences.

The retinal pigment epithelium is anatomically and functionally closely related to the photoreceptor layer, and its function is of

crucial importance to visual function. Acute loss of the structural integrity of the pigment epithelium leads within a few days to the degeneration of the photoreceptor cells. However, only little is known about the exact processes in which the pigment epithelium is involved (Moyer, 1969). Electron microscopic observations suggest that intensive transport by means of pinocytosis takes place over the cell, but it is not sure in which direction this occurs. Probably the pigment epithelium, interposed between the blood supply from the choriocapillaris and the photoreceptor cells, has a nutritive function to the photoreceptors. This is supported by the electron microscopic observation that the heavily plicated basal surface of the pigment epithelial cells is in close contact with the endothelial surface of the choriocapillaris which shows extensive fenestrations. However, the nature of the nutritional functions are largely unknown.

There are observations which suggest that the pigment epithelium is the major source of the resting potential, measured across the intact eye and active transport of chloride ions from the photoreceptor side to the scleral side of the pigment epithelium has been observed. The role of this potential in vision is not known.

At least part of the mucopolysaccharides, filling the space between the photoreceptors and the pigment epithelium is produced in the pigment epithelial cells, but the function of this polysaccharide matrix is not clear.

The role of the pigment epithelium in the vitamin A movements in the eye during light and dark adaptation (Dowling, 1960) and its phagocytotic activities towards rod outer segments (Berman, 1971) are elucidated only to a limited extent. Biochemical defects in processes as mentioned undoubtedly have adverse effects not only to the pigment epithelial cells, but result also in malfunctioning of the photoreceptors. In order to understand the basic pathology of tapetoretinal degenerations it is important to know what are the normal metabolic pathways in the photoreceptor layer and its adjacent structures.

2.2 Photoreceptor cells

2.2.1 The rod photoreceptor

The rods are differentiated into two major divisions, an outer

segment and an inner segment. The rod outer segment is the photosensitive part of the rod. It is composed of a cilindrical stack of flat sacs or discs, surrounded by an outer membrane, each disc consisting of a double membrane layer, in which the visual pigment rhodopsin is embedded. The disc membranes are directed perpendicular to the direction of the incident light, providing optimal conditions for light absorption. The outer segment depends for its continual synthesis and its metabolism on the inner segment. All cell organelles involved in cell metabolism, such as mitochondria, Golgi complex and endoplasmic reticulum, are located in the uppermost part of the inner segment close to the connecting cilium, which connects the outer and the inner segment (Young, 1967).

The rod photoreceptor membrane is one of the best studied biological membranes. On a dry weight basis cattle photoreceptor membranes consist for at least 40% of lipids. About 80% of these lipids are phospholipids, mainly phosphatidylethanolamine, phosphatidylcholine and phosphatidylserine. The fatty acids in the lipids are mostly highly unsaturated resulting in a very fluid type of the membrane (Borggreven et al., 1970). Approximately 50% of the membrane consists of protein. As much as 85% of the total membrane protein is visual pigment rhodopsin. The remaining membrane protein comprises a number of enzyme activities. The purity of the membrane material influences the number of enzymes found. Some enzymes that were said to be related with rod outer segment membranes, are not present in better purified preparations. The following enzymes are reported to be related with rod outer segment membranes: retinoldehydrogenase (Bridges, 1962; Futterman, 1963; de Pont et al., 1970; Kissun et al., 1972), Na-K-activated ATPase, Mg-activated ATPase (Bonting et al., 1964; Frank et al., 1973), guanylate cyclase (Pannbacker, 1973, 1974; Bensinger et al., 1974), cyclic nucleotide phosphodiesterase (Pannbacker et al., 1972; Bitensky et al., 1973; Chader et al., 1974a, 1974b; Lolley and Farber, 1975). These enzymes may be involved in the photoreception process.

2.2.2 Biosynthesis of rod outer segment membranes

The biosynthesis of rod outer segment membranes have been elucidated by Young and co-workers, mainly by means of autohistoradiography in

various species like monkey and frog (Young, 1974; Bibb and Young, 1974a, b). Outer segment membrane is continuously formed at the base of the rod outer segment. The proteins and at least the glycerol component of the phospholipids which are destined to form the outer segment membrane, are produced in the inner segment of the visual cell. Autohistoradiographic investigations have shown that proteins, including opsin, are produced in the myoid portion of the cell inner segment. Most or all of the proteins destined for the outer segment then pass through the Golgi complex before traversing the connecting cilium. Presumably all membrane components are transported from the inner to the outer segment by way of the hollow cilium, which connects these two parts of the cell. Once the proteins have been built in a disc membrane of the rod outer segment they stay unaltered and move upward to the pigment epithelium where digestion of the membranes take place. In contrast, the phospholipids in the disc membranes are subject to rapid exchange processes especially the fatty acids in the phospholipids. The glycerol part of the phospholipids is exchanging to a lesser extent than the fatty acids. This indicates that the rods renew their membrane lipids by both molecular replacement and fatty acid exchange. The final assembly of the membrane constituents into a membrane probably takes place in the outer segment. The outer segment discs are formed by the repeated infolding of the outer cell membrane. The invaginations are then transformed into discs which are not in contact with the outer membrane.

The site at which vitamin A aldehyde is added to the visual pigment protein is unknown. Autoradiographic studies reveal that ^{3}H-retinaldehyde is not initially concentrated in the visual cell inner segment. Instead it is bound immediately in the outer segment. The incubation of fully bleached retinas in the light does not impede the incorporation of new opsin molecules into growing outer segment membranes. This suggests that opsin can be inserted into the membrane whether or not retinaldehyde is attached.

2.2.3 Digestion of rod outer segment membranes

Rod discs are continuously detached from the top of the outer segment and are degraded in the pigment epithelium. Broad villi of the pigment epithelium form bowl-like sheats around the photoreceptors and

play a role in the phagocytosis of their tips. They form cytoplasmic protrusions extending on all sides of the tip of the outer segment to sequester 30 to 40 discs. The protrusions fuse to enclose the uppermost 30 - 40 discs and their surrounding cell membrane in the epithelial cell cytoplasm. These cytoplasmic inclusions are known as phagosomes. The phagosomes are surrounded by the cell membrane of the photoreceptor. This in turn is surrounded by another cell membrane that is derived from the pigment epithelial cell. Lysosomes fuse with the phagosome and digestion takes place until only some lipofuscin granules are left (Spitznas and Hogan, 1970). The visual pigment rhodopsin is also degraded. The chromophoric group is probably recycled for use in the rhodopsin synthesis at the basis of the rods.

As a result of the continuous renewal and digestion of rod outer segment membranes, in mammals approximately 10% of the membranes is replaced each day. Consequently each day 10% of the total rhodopsin in the eye is degraded in the pigment epithelium. Vitamin A compounds are involved in this metabolic rod renewal cycle, being continuously incorporated into the outer segment membranes at the base and being removed in the pigment epithelium.

2.3 The visual pigment rhodopsin

Rhodopsin is the main component of the disc membrane. The properties of rhodopsin are extensively studied, especially in cattle. It is a lipoprotein associated with membrane phospholipids. It is insoluble in aqueous media and relatively resistant to physical and chemical influences. Its properties have mostly been studied in an aqueous environment, after solubilization with the aid of detergents. In these detergents the membrane fragments are solubilized by incorporation into micelles. The spectral properties of rhodopsin do not change upon solubilization. However, other parameters, such as reaction kinetics may change. Other components of the disc membrane, e.g. enzymes, may be altered in their properties by detergents.

The characteristic absorption spectrum of rod photoreceptor membranes solubilized in detergent solution (digitonin) in darkness is presented in fig. 6.

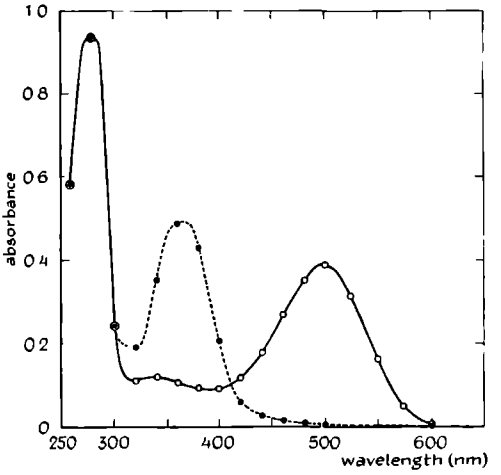

Fig. 6 Absorption spectrum of rhodopsin in 1% digitonin
——— before illumination
------- after illumination in the presence of hydroxylamin

It shows the three main absorption bands with peaks at 500 nm (alpha band), 340 nm (beta band), and 278 nm (gamma band). The γ-band is the typical protein absorption band. The α- and β-bands are characteristic for rhodopsin. They disappear upon illumination, making way for a new absorption band around 380 nm, arising from the liberated chromophoric group. In the presence of hydroxylamine which binds retinaldehyde as an oxime, this band shifts to 360 nm. Since the latter band barely absorps at 500 nm, the difference in absorbance at 500 nm before and after illumination is proportional to the amount of rhodopsin originally present, and thus serves as a usefull analytical parameter.

2.3.1 Chromophoric group

Rhodopsin owes its characteristic properties to the presence of a very lipophylic chromophoric group, retinaldehyde, the aldehyde derivative of vitamin A. Such polyenic molecules may exist in various *cis-trans* stereo-isomers. The isomeric form present in rhodopsin was first identified as the *11-cis* isomer by Hubbard and Wald (1952 a, b). More definitive proof has been provided by Rotmans et al. (1972a). The *11-cis* isomer is thermodynamically the least stable of the various known

stereo-isomers. Fig. 7 shows its structure.

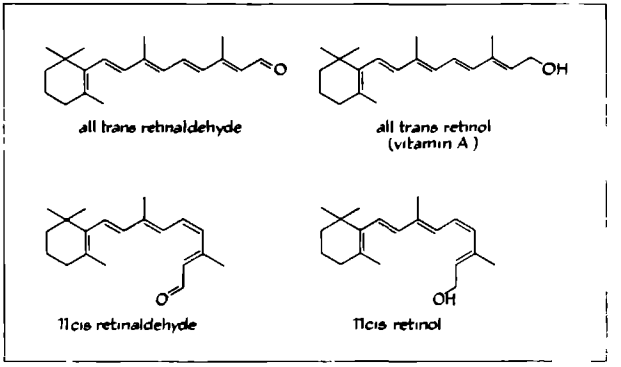

Fig. 7 The various vitamin A compounds present in the vertebrate eye

The chromophore in rhodopsin is linked to an aminogroup of opsin in the photoreceptor membrane under formation of a protonated Schiff base. The binding site in rhodopsin has been identified as the ε-amino group of a lysine residue of opsin (de Grip et al., 1973).

2.3.2 Photolysis

Light has a catalytic action on rhodopsin. Absorption of light results in excitation of the chromophoric group *11-cis* retinaldehyde, which thereupon isomerizes to the *all-trans* isomer (Hubbard, Wald, 1952a, b). This isomerization step, which is the only step requiring light, induces a protein-conformational change, leading to a series of thermal reaction steps resulting ultimately in visual excitation. These steps are reflected in definite spectral changes. Eventually rhodopsin desintegrates into its components: opsin and *all-trans* retinaldehyde. At some point during this reaction visual excitation is induced.

2.4 Visual excitation

Upon illumination of the human retina with normal light intensities, an electrical response is observed, called the electroretinogram. It represents a potential wave, beginning with a sharp negative deflection, the a-wave, followed by two positive components, the b-wave and the

c-wave. The a-wave arises from the photoreceptor cells. Bipolar, horizontal and Müller cells contribute to the b-wave. The c-wave originates in the pigment epithelium cells. The contribution of the rods to the a-wave appears to arise from a light-induced decrease in a rod dark current. There exists an intensive dark current of sodium ions along the entire rod, passing through the plasma membrane of the inner segment in the outward direction and entering via the plasma membrane of the outer segment (Hagins et al., 1970; Hagins, 1972). This dark current is probably maintained by a Na-K-activated ATPase system in the cell membrane of the inner segment. Illumination decreases the relatively high sodium permeability of the outer segment plasma membrane, resulting in a reduction of the dark current and a hyperpolarization of the cell.

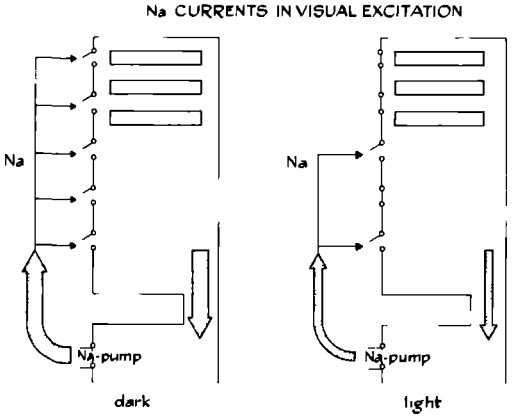

Fig. 8 Schematic representation of the sodium current in the vertebrate rod

How the photolysis of rhodopsin at the disc membrane can lead to a decrease of the sodium permeability of the outer segment plasma membrane may be explained by the hypothesis that calcium ions act as a transmitter between the discs and the plasma membrane of the outer segment (Hagins, 1972; Yoshikami and Hagins, 1973). Photolysis of rhodopsin would release calcium ions, stored in the rod disc, to the cytoplasm, which then diffuse to the outer membrane where they block the sodium channels. The dark current of sodium ions along the rod seems to keep the synapse of the rod activated. The synaptic end of the rod, the sphe-

rule, presumably responds by continuously releasing a transmitter substance, which keeps the horizontal and bipolar cells depolarized (Dowling and Ripps, 1973). During illumination, the sodium current decreases as a result of the reduction of the sodium permeability of the outer segment membrane. This leads to a decrease in transmitter release at the synaps and consequently to a hyperpolarization of horizontal and bipolar cells. This impulse is then transmitted to the ganglion cells and the central nervous system.

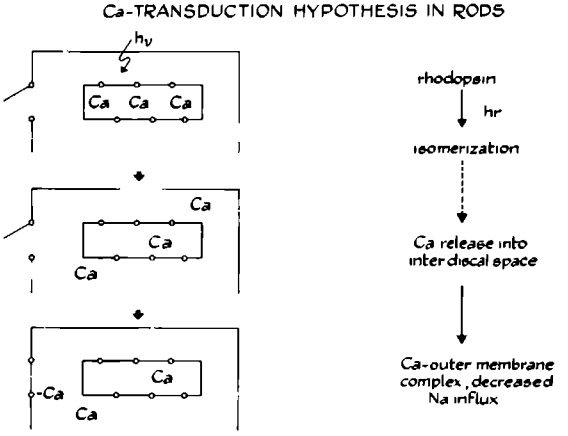

Fig. 9 Schematic presentation of the calcium transmitter hypothesis

2.5 The regeneration process

2.5.1 Regeneration of visual pigment

After illumination and bleaching of rhodopsin, reconstitution of rhodopsin must take place to retain visual sensitivity. This process is called regeneration. In vivo this process has been investigated in man by retinal densitometry, a method in which the light reflected by the fundus oculi is measured. Measurements with densitometry on the parafovea show that after a full bleach rhodopsin regenerates along an exponential curve with a half time of about 4 minutes. Regeneration is complete in 30 - 60 minutes.

In vitro, regeneration can be accomplished by addition of the chromophoric group, *11-cis* retinaldehyde, to opsin. In vivo, the last step in the regeneration process is presumably the same: recombination of

opsin with *11-cis* retinaldehyde. This means that there must exist in the retina a metabolic pathway that isomerizes *all-trans* retinaldehyde, liberated upon illumination, to the *11-cis* form. No such pathway has been found so far, although the possible involvement of a reduced flavin catalyst has been discussed by Futterman and Rollins (1973).
Futterman and Futterman (1974) found that *11-cis* retinaldehyde can be effectively isomerized by dihydroriboflavin and other nucleophiles in aqueous solution. However *11-cis* retinaldehyde cannot be detected at equilibrium among the products, but the ease with which *11-cis* retinaldehyde reacts in these circumstances makes them suggest the possibility that the in vivo conversion of *all-trans* retinaldehyde to *11-cis* retinaldehyde occurs by an enzymatic variant of nucleophilic catalysis.

The problem of regeneration has been studied in different ways. A number of authors have studied regeneration in isolated retinas, mostly of rats and frogs (Crescitelli and Sickel, 1968; Weinstein et al., 1967; Goldstein, 1967: Baumann, 1965). Although electrical responses (ERG) could be elicited, no regeneration of visual pigment occurred. However, frog retinas in contact with pigment epithelium are able to regenerate their rhodopsin (Kühne, 1879; Reuter, 1966) and homogenates of frog and rat retinas and pigmented layers show regeneration of rhodopsin in the presence of *all-trans* retinol (Collins et al., 1953). Cone and Brown (1969) found under special conditions regeneration of visual pigment in the isolated rat retina. The retina, isolated from dark adapted rats was suspended in a small volume of Ringer solution in a chamber only slightly greater than the retina itself, maintained at 37°C and an area of 1 - 3 mm diameter was irradiated with 510 - 1000 nm light. Regeneration of 80% of the visual pigment could then be detected, as shown by an increase of spectral absorption at 500 nm. Other investigators too have found the formation of *11-cis* retinaldehyde in the isolated rat retina (Amer and Akhtar, 1973a) and in the isolated frog retina (Amer and Akhtar, 1973b). However, although they give evidence that *11-cis* retinaldehyde is formed from *all-trans* retinaldehyde, they do not show that rhodopsin is actually formed.

2.5.2. The visual cycle

Another way of elucidating the visual pigment regeneration process

is to investigate the distribution of vitamin A compounds in the retina during light and dark adaptation and to study the enzymes involved in vitamin A metabolism in the retina. According to Wald (1968) the visual cycle of the vitamin A compounds may be hypothetically depicted as in fig. 10.

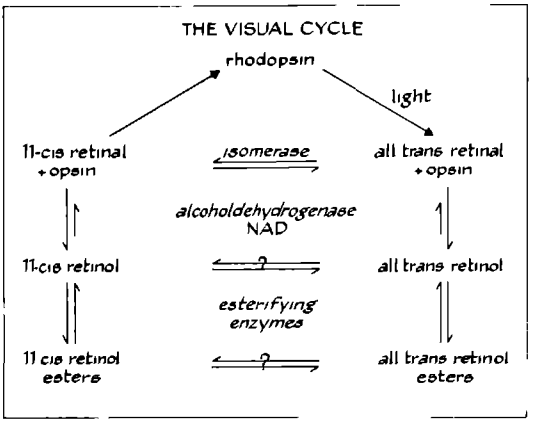

Fig. 10 The classic rhodopsin cycle (Wald 1968)

Knowing the distribution of the various vitamin A compounds in the eye during light and dark adaptation, should help towards testing this hypothesis. This distribution has been studied in the albino rat by Dowling (1960). The total amount of vitamin A compounds in the eye does not vary during light and dark adaptation. During light adaptation the retinaldehyde content of the eye falls, as the retinaldehyde liberated on bleaching of rhodopsin, is reduced to retinol. The retinol level in the retina increases for some time and then declines, when the bulk of the retinol moves to the pigment epithelium. During dark adaptation these processes are reversed. The retinaldehyde content of the eye increases as rhodopsin is formed while the retinol level in the pigment layer and retina declines. Return to the dark adapted situation is completed in about 100 - 120 min.

Krinsky (1958) has demonstrated the esterification of vitamin A in the eye tissues of cattle and has found that much of the retinol, both in the retina and in the pigment epithelium is esterified. He also demon-

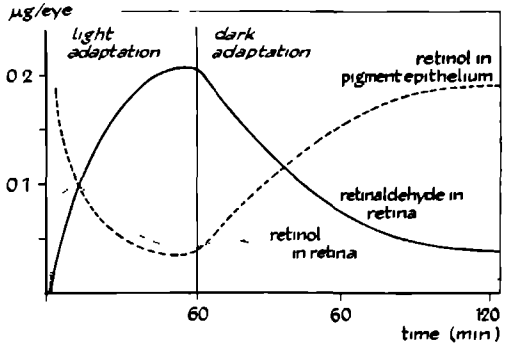

Fig. 11 Distribution of vitamin A compounds in the albino rat eye during light adaptation and dark adaptation (Dowling, 1960)

strated that as much as 65% of the total vitamin A ester in the cattle pigment epithelium might be in the *11-cis* configuration. The esterifying enzyme displayed no preference for *all-trans* or *11-cis* retinol.

Hubbard and Dowling (1962) have studied the concentration of *11-cis* vitamin A compounds in frog eyes during light and dark adaptation. In darkness about 42% of the total vitamin A, not bound in rhodopsin, is stored in the *11-cis* configuration. In the light adapted eye the proportion of *11-cis* isomer is only about 25%. During subsequent dark adaptation, which takes about 2 hrs, this proportion stays constant. This means that an isomerizing system must be present keeping the relative proportions of *11-cis* and *all-trans* vitamin A compounds constant, while *11-cis* retinaldehyde is being bound in rhodopsin.

The results of Dowling (1960) have been confirmed and extended by Zimmerman. He measured the amount of *11-cis* and *all-trans* vitamin A compounds during light and dark adaptation in the rat. During light adaptation the amount of *11-cis* retinaldehyde in the eye, most of which is bound in rhodopsin, decreases, while *all-trans* retinaldehyde increases. *All-trans* retinaldehyde is subsequently reduced to *all-trans* retinol and is esterified, partly in the retina itself and partly in the pigment

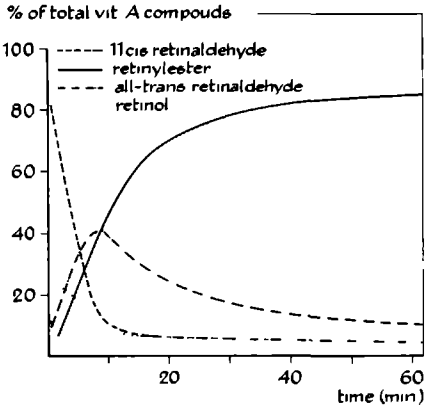

Fig. 12 Proportions of vitamin A compounds in the eye during light adaptation (Zimmerman, 1974).

epithelium. This process reaches an equilibrium in about 60 min. During dark adaptation the amount of *11-cis* retinaldehyde increases, reflecting the re-formation of rhodopsin, while the retinyl ester level in the pigment epithelium decreases. This process takes about 5 hrs.

Probably these processes serve to confine the chromophore vitamin A in the eye and to recycle vitamin A in the rhodopsin regeneration. During light adaptation the reduction of liberated chromophore to retinol and the subsequent esterification may serve as an overflow mechanism, which prevents accumulation of free vitamin A compounds that might be damaging to cell membranes. Zimmerman et al. (1974) also gave evidence that after a small bleach of rhodopsin in the rat retina in vivo the increase of *11-cis* retinaldehyde occurred at least in part from re-isomerization of *all-trans* retinal, without prior reduction to retinol. It has not yet been settled whether after scotopic illumination regeneration takes place from retinaldehyde present in the receptor cell or whether a longer metabolic pathway via the pigment epithelium is involved. In fact, although the pigment epithelium plays an important role in the metabolism of rhodopsin in the outer segment, it is not clear whether close contact between photoreceptor layer and pigment epithelial layer is essential for rhodopsin regeneration. To answer this question

experiments on the regeneration of the isolated rat retina as described by Cone and Brown (1969) should be valuable.

2.6 Retinoldehydrogenase activity in the retina

The conversion of retinol to retinaldehyde and vice versa is a reaction catalyzed by alcoholdehydrogenases with NAD or NADP acting as cofactor. The retina contains retinoldehydrogenase activity (Wald, 1949, 1950; Futterman and Saslaw, 1961; Futterman 1963, 1965; Koen and Shaw, 1966; Reading and Sorsby, 1966; de Pont, Daemen and Bonting, 1970). In view of the visual cycle as proposed by Wald it is important to know the exact localization of alcoholdehydrogenases in the retina. Also with respect to the localization of an isomerizing mechanism, it is important to know whether these alcoholdehydrogenases show a certain substrate specificity, particularly a specificity to the stereo-isomers of vitamin A compounds. There is a narrow functional relationship between photo-receptor cells and pigment epithelium in the vitamin A metabolism in the retina, both in the renewal cycle and in light and dark adaptation. Thus it might be very interesting to look at retinoldehydrogenases in the pigment epithelium, the presence of which has been shown already in the rat retina by Kissun, Graymore and Newhouse (1972).

CHAPTER 3

RETINITIS PIGMENTOSA

3.1 Introduction

Retinitis pigmentosa is a retinal disease in man with a hereditary etiology. On the basis of heredity three types of retinitis pigmentosa can be discriminated: autosomal dominant, autosomal recessive and x-linked. About 20% of the patients with retinitis pigmentosa are sporadic cases, many of which probably belong to the autosomal recessive group (Jay, 1972). The pathogenesis is still unknown. The disease runs a chronic and progressive course and frequently results in blindness, due to a degeneration of the photoreceptor layer and the pigment epithelial layer. The disease can be classified clinically in the group of hereditary tapetoretinal degenerations (Carr, 1972). These tapetoretinal degenerations are a group of hereditary retinal diseases, caused by unknown metabolic defects in one or more retinal layers. Retinitis pigmentosa is a typical example of a hereditary tapetoretinal degeneration, which occurs fairly frequent. Although the symptoms of even this fairly circumscript syndrome may vary widely, a classical picture of the disease can be given.

The first symptom noted in an early stage is night-blindness. As the disease progresses, characteristic abnormalities develop which include: (1) attenuation of the retinal vessels, (2) waxy pallor of the optic disc and (3) pigment aggregations in the retina with depigmentation of the pigment epithelium. The pigment accumulation starts first in the mid-periphery of the retina by the formation of clusters resembling bone corpuscules around the retinal vessels. In the end the whole retina becomes studded with pigment aggregations, while the pigment epithelial layer has largely disappeared. The vitreous contains cellular elements.

The visual field is characterized by the development of a ring-shaped scotoma, corresponding to the retinal degenerative changes. Central vision stays intact for a long time, and is the last function to be affected. Colour vision is affected in the later stages of the disease. Most often blue-yellow perception is disturbed. Dark adaptation is delayed, the ERG is diminished or abolished. The EOG shows alte-

rations even before the ERG diminishes.

The pathology of the disease essentially consists of a degeneration of the retinal neuro-epithelium and the pigment epithelium, suggestive of a metabolic disorder in the photoreceptor cells or the pigment epithelium. First the rod photoreceptor cells degenerate, and soon degeneration of the pigment epithelial cells becomes manifest. Migration of pigment granules from the pigment epithelium into the retina is responsible for the characteristic fundus aspect. The cause of the disease appears to be a biochemical lesion in the pigment epithelium-photoreceptor complex, but the precise etiology is not yet known. It is very probable that different abnormalities in this complex can account for the changes that we see in retinitis pigmentosa.

There are other hereditary tapetoretinal degenerations, which can be discriminated from retinitis pigmentosa by their different clinical symptoms, by the different clinical course and by the different mode of heredity. These also result in a degeneration of the photoreceptor layer and the pigment epithelium, and thus the clinical symptoms in long standing disease in most tapetoretinal degenerations are very similar. This makes a clinical classification of patients with hereditary tapetoretinal degeneration difficult. Clinical research in patients with a hereditary tapetoretinal degeneration, without presentation of all relevant clinical data to ensure that the group is homogeneous, is only of limited value. It is also wrong to think that a tapetoretinal degeneration in an experimental animal is representative for retinitis pigmentosa in man. It can only be used as an experimental model for the study of tapetoretinal degenerations.

Although the clinical classification of many hereditary retinal diseases is being increasingly clarified and the information about physiology and biochemistry of retinal metabolism is growing steadily, only very little is known about the metabolic site of the initial pathology in these disorders. From histological and biochemical data we know that there exists a very delicate relationship between the different retinal layers, especially the photoreceptor layer, the retinal pigment epithelium, Bruch's membrane and the choriocapillaris. A disturbance in one layer nearly always has a deleterious effect on the other layers. So,

when the pathology of these diseases is studied in late stages, they are all remarkably similar. This does, of course not mean that the original defect is the same in all hereditary retinal diseases. Because eyes of patients with hereditary tapetoretinal degenerations are rarely available for biochemical investigations, biochemical research has largely been restricted to animal experiments.

3.2 Clinical data

The only clinical data on the possible etiology of hereditary tapetoretinal degenerations in man concern abnormalities of vitamin A metabolism and some immunologic observations.

3.2.1 Vitamin A metabolism

It is known, both clinically and experimentally that a severe vitamin A deficiency results in a degeneration of the rod photoreceptors (Dowling, 1964; Rabin et al., 1973; Hayes, 1974). After prolonged vitamin A deficiency the rod photoreceptor layer and the pigment epithelium degenerate, resulting in an ophthalmoscopic appearance more or less similar to other tapetoretinal degenerations. In patients with a-β-lipoproteinemia, serum vitamin A levels cannot be maintained, because intestinal absorption and transport of vitamin A is disturbed, resulting in tapetoretinal degeneration (Gouras et al., 1971). In Refsum's disease the deposit of an abnormal lipid in the retina may interfere with the exchange of vitamin A compounds in the various retinal layers (Toussaint and Danis, 1971). A well documented group of patients with genetically determined isolated retinitis pigmentosa has been examined by Massoud et al. (1975) for blood plasma vitamin A and β-carotene levels. They failed to find any difference between the group of patients and a control group.

In recent papers much attention has been focussed on a serum protein which is a carrier of vitamin A, the retinol binding protein. Abnormalities in level or structure of retinol binding protein might be a cause of tapetoretinal degeneration. Although quantitative differences in patients with "retinitis pigmentosa" compared to normal individuals have been claimed (Rahi, 1972), this has been contradicted by other

authors (Gouras and Chader, 1974; Maraini, 1974; Futterman et al., 1974). At present only one publication exists in which a well documented group of patients with various confirmed hereditary tapetoretinal degenerations has been examined for blood levels of retinol binding protein. (Maraini et al., 1975). In this study, none of the patients showed a serum level of retinol binding protein differring significantly from control subjects.

3.2.2 Immunologic data

In a publication of Rahi (1973) serum immunoglobulin levels have been examined in an ill-defined group of patients with "retinitis pigmentosa". Although serum IgM levels were raised in part of the patients compared to a control group, the IgM levels did not exceed the normal range. Char et al. (1974) showed in a small heterogeneous group of patients with retinal pigment degenerations that high cell-mediated immunity was present against a tissue culture from retinoblastoma, measured by cytotoxic effect. They presume that this might well be a secondary event as a result of sensitization against antigens released from the diseased retinal cell.

3.3 Experimental data

Experimental data are largely restricted to animal experiments with a strain of rats with a hereditary retinal degeneration and with normal rats with experimentally induced tapetoretinal degeneration. Also a mouse strain with a selective degeneration of the retinal photoreceptor layer has been investigated.

3.3.1 Hereditary retinal degeneration in the rat

All authors have used descendants of a rat strain first described by Bourne et al. (1938). The animals show an autosomal recessive retinal degeneration which has some resemblance with tapetoretinal degeneration in man. The rapid onset of symptoms in these animals has its equivalent in man in Leber's congenital amaurosis.

Histology

Dowling and Sidman (1962) have described the histological changes

of retina and pigment epithelium in these rats in detail, and have correlated them with changes in the electroretinogram.
Rod outer segments develop normally in the beginning, but after a few days the rod outer segment layer becomes much wider than in control animals and contains large lamellar whorls between the outer segments and the pigment epithelium. The rhodopsin content of the eye also becomes much higher than normal, reaching a maximum after about 30 days when the animals are kept in darkness. From 22 days onwards the production of outer segments gradually ceases, and the inner segments degenerate. At the age of 40 days degeneration of the pigment epithelium becomes manifest. After 1 year the outer segments have disappeared, and intact layers of bipolar and ganglion cells are adjacent to a dedifferentiated pigment epithelial layer covering Bruch's membrane.

Bok and Hall (1969) have shown by means of autohistoradiographic methods that the lamellar material piling up between photoreceptors and pigment epithelium consists of rod outer segment membrane material. La Vail et al. (1972) gave evidence with the same technique that membranes of pigment epithelial processes also take part in the formation of lamellar whorls. However, more recent findings (LaVail and Batelle, 1975) suggest that the lamellar whorls are entirely formed from the breakdown of rod outer segments. Bok and Hall (1971) have also shown that, although ingested rod outer segment membrane lamellae called phagosomes are clearly present in the pigment epithelium of normal animals, there are no phagosomes present in the pigment epithelium of rats with inherited retinal dystrophy. Herron et al. (1969, 1971) and Custer and Bok (1975) have confirmed the lack of phagosomes in the pigment epithelium of the dystrophic rat. They also conclude that the production of rod outer segments in the afflicted animals is not increased compared to control animals.

The general conclusion from these histologic data is that the normal continual digestion of rod outer segment material by pigment epithelial cells does not take place in these animals. This could be due to a conformational change of the rod outer segment membranes or to a defect in the pigment epithelial cell, either of which precludes digestion of the rod outer segment membranes.

Biochemistry

Other experimental studies with rats with inherited retinal degeneration concern biochemical abnormalities resulting in a disturbed relationship between rod outer segments and pigment epithelium. Although several abnormalities in retinal metabolism in rats with inherited retinal degeneration have been claimed, a clear causal relation with the anatomical changes, the accumulation of whorls of rod outer segment membranes between the rods and the pigment epithelium and the absence of phagosomes in the pigment epithelium, cannot be established.

In general, the metabolism of the dystrophic retina seems disturbed only secondary to the accumulation of rod outer segment membranes. Protein and amino-acid metabolism in the retina of the dystrophic rat is normal (Reading, 1970). The protein synthesis in the retinal pigment epithelium is reported to be elevated (LaVail et al., 1972) when rod outer segment membranes are piling up next to it.

Carbohydrate metabolism does not differ from control animals (Reading 1970; Ponte et al., 1974), although the hexose monophosphate shunt activity in the retina of rats with hereditary retinal degeneration is elevated. However, this metabolic pathway is coupled via its co-enzyme NAD(P) with retinoldehydrogenase activity. Due to the large amounts of rhodopsin in the dystrophic retina, bleaching results in a release of high quantities of *all-trans* retinaldehyde. This retinaldehyde is reduced to retinol by retinoldehydrogenase and in this reaction NAD(P)H is oxidized to $NAD(P)^+$. $NAD(P)^+$ is then reduced in the hexose monophosphate oxidation pathway resulting in elevation of its activity. The high concentration of retinol released in this process may well have a deleterious effect on the retinal tissues by disrupting the lysosomes present in the pigment epithelium, resulting in a release of hydrolytic enzymes in the retinal tissues (Burden et al., 1971; Vento and Cacioppo, 1973; Dewar et al., 1975a). Only one abnormality in carbohydrate metabolism has been claimed that may precede the accumulation of whorls of rod outer segment membranes. In normal rat retinas the isoenzyme pattern of lactic dehydrogenase activity shows two major fractions, the "M" (muscle) and "H" (heart) isoenzymes (Graymore, 1964 a, b). Bonavita et al. (1963) and Graymore (1964 a, b) showed an anomalous isoenzyme pattern for lactic dehydrogenase in retinas of rats with inherited retinal degeneration, the

"M" type being deficient from birth. It is not clear if this is a coincidental finding or is concerned with the basic pathology of the retinal degeneration.

No abnormalities have so far been found in the composition of the rod outer segment membrane that could prevent ingestion by the pigment epithelium. Rhodopsin formed in rats with hereditary retinal degeneration does not differ in its chemical and physical properties from rhodopsin in normal animals (Dowling and Sidman, 1962). Na-K-Mg-activated ATPase, an enzyme closely attached to the rod outer segment membranes was reported by Bonavita et al. (1966, 1967) to be progressively deficient in the dystrophic rat retina, but experiments described in this thesis (chapter 8.3) gave evidence that ATPase activity, both Mg- and Na-K-Mg-activated in retinas of afflicted animals does not differ from that in normal animals.

Although there is ample histologic evidence that there is no digestive activity of the pigment epithelium towards rod outer segment material, histochemical studies have shown that there are lysosomes present in the pigment epithelial cell, and no abnormalities in lysosomal enzymes have been found (Bok and Young, 1972; Burden et al., 1971; Feeney, 1973; Ansell and Marshall, 1974).

Investigations into the retina metabolism of a mouse strain with an autosomal recessive mutation which causes a selective degeneration of the photoreceptor layer of the retina revealed a deficiency in cyclic nucleotide phosphodiesterase activity (Schmidt and Lolley, 1973; Farber and Lolley, 1973), and an elevation in cyclic GMP levels (Farber and Lolley, 1974) apparent before the photoreceptor cells begin to degenerate. These findings led Lolley and Farber (1975) to investigate a possible dysfunction in cyclic GMP metabolism in rats with inherited retinal degeneration. They found that for the first 14 days of life the kinetic characteristics of cyclic guanosine monophosphate phosphodiesterase in the dystrophic rat retina are similar to those of control retinas. Thereafter the kinetics and activities change, probably due to interaction with material derived from the accumulation of rod outer segment material. Dewar et al. (1975b) found that cyclic AMP phosphodiesterase activity in dystrophic rat retinas was deficient preceding the onset of photoreceptor degeneration. Since Lolley et al. (1974) found elevated

cyclic AMP-concentrations primarily in the inner retinal layers of dystrophic animals, the connection with disturbed phagocytosis by the pigment epithelium is not clear. The role of cyclic nucleotides in rod outer segment metabolism is not yet settled. This makes the relevance of the observations on cyclic nucleotide metabolism in dystrophic animals difficult to assess.

Recently a new form of hereditary retinal degeneration in a rat strain has been described by Yin Lok Lai et al. (1975). This retinal disease is characterized by a slow progressive degeneration of the photoreceptor layer. The earliest changes were visible at the age of one month in the photoreceptor inner segment, suggesting that the primary lesion is located there.

3.3.2 Experimentally induced tapetoretinal degeneration

A tapetoretinal degeneration can be experimentally induced in normal rats by means of a nutritional vitamin A deficiency (Dowling, 1964), or by means of long term illumination at high light intensities (Kuwabara, 1970; Noell et al., 1971). Vitamin A deficiency causes a degeneration of rod photoreceptors probably because rhodopsin, one of the main constituents of the rod outer segment membrane, cannot be formed.

Vitamin A deficiency

Recent publications of Herron and Riegel (1974 a, b) concern controlled degeneration of rod outer segments in normal rats by means of a slight vitamin A deficiency. They have found that in rats placed on a vitamin A deficient diet, supplemented with retinoic acid, the thickness of the rod outer segment layer is decreased, while the time interval between production and removal of rod outer segment membranes remained constant. After restoring the normal diet the amount of rhodopsin present stays subnormal. This means that temporary administration of a vitamin A deficient diet may result in a permanent decrease in the production of rod outer segment membranes. If the retinal degeneration in the rat strain with hereditary retinal degeneration is caused by impaired digestive activity of the pigment epithelium, a controlled degeneration of rod photoreceptors could reestablish the equilibrium between production and removal.

Their results in normal rats are surprising because it is known from experiments of Dowling (1964) that the retina is extremely resistant to vitamin A deficiency. However, it would be interesting to see the results of similar experiments carried out with rats with hereditary retinal degeneration. Such experiments have not yet been published.

Influence of light.

High light intensities are damaging to the retina of albino rats and prolonged illumination results in a retinal degeneration, which in its final stage resembles hereditary tapetoretinal degeneration (Kuwabara, 1970). The presence of light, oxygen and a high concentration of pigment creates favourable conditions for the formation of radicals, which can lead to deleterious chemical reactions in proteins and unsaturated lipids (Daemen, 1973). Rod outer segment membranes have a very high content of long chain highly unsaturated fatty acids. Noell and Albrecht (1971) suggest that this may be an explanation for the fact that illumination is damaging to the retina. Anti-oxidant systems which can prohibit this damage are present in the normal retina. Levels of ascorbic acid and tocopherol are high in rod outer segments, at least in cattle (Daemen, 1973). Futterman (1963) showed that the pentose phosphate pathway, the primary function of which is the generation of NADPH, is active in cattle rod outer segments. Whether a defect in these anti-oxidant systems plays a role in some forms of hereditary tapetoretinal degenerations is not known.

The albino rat, which is a nocturnal rodent deficient in pigmentation is probably the least suitable experimental animal for studying the influence of light on retinal degeneration. Recently, pigmented rats with hereditary retinal degeneration have been obtained and have been studied with respect to ophthalmoscopic appearance and histology (Herron et al., 1974; Yates et al., 1974). Although the histological changes are the same as in the albino rat strain, the influence of illumination on the disease can be studied in a more reliable way. Yates et al. (1974) conclude from histologic and biochemical observations that retinal degeneration in pigmented animals is delayed, probably due to the presence of melanin pigment in the eye.

3.4 Conclusion

Concerning the basic pathology of hereditary tapetoretinal degenerations many problems have yet to be solved. The clinical classification of hereditary retinal diseases is still very difficult, mainly as a result of the similarity in symptoms in long standing disease. It is impossible to draw reliable conclusions from biochemical findings in a heterogenous group of patients such as the retinitis pigmentosa group. Therefore clinical diagnostics can help research by discriminating as accurate as possible the various tapetoretinal degenerations.

A serious problem in clinical research is that the biochemical substrate, i.e. retinal tissue of very young patients with little or no retinal degeneration, is not accessible for biochemical investigation methods. This problem can only be solved by investigations in experimental animals, although the results may be of limited value for human pathology. By investigating the normal metabolic pathways of retinal photoreceptor metabolism and correlating the results with clinical symptoms, it may be possible in the future to recognize the metabolic errors that are responsible for various tapetoretinal degenerations.

An important factor in the relationship of photoreceptors and pigment epithelium is the vitamin A metabolism. Our experimental findings in normal vitamin A metabolism in cattle, which are described in chapter 6, suggest another enzyme that might be tested for involvement in tapetoretinal degeneration in the rat, a particulate retinoldehydrogenase present in the pigment epithelium, which is able to convert *11-cis* vitamin A compounds. Many problems in vitamin A metabolism are still unanswered. Pigment epithelial function, e.g. the degradation of rod outer segment membranes is still not understood.

Lipid metabolism of retinal photoreceptors has scarcely been investigated. The metabolic events of membrane lipids may be very important for the function of the photoreceptors, particularly in respect of resistance to photic damage.

By solving these problems the biochemist may give the clinician the key to the various causes of tapetoretinal degenerations.

3.5 Aims of this thesis

The preceding introduction shows that the cause of many hereditary retinal diseases are still completely unknown, although it is clear that metabolic dysfunctions underlie this group of retinopathies. The fact that in most cases only the retina is diseased and that the patients are healthy otherwise, has several consequences. Many patients with a hereditary retinal degeneration eventually become blind, while they are still in good general health. This is a heavy burden for the patient and his surrounding. It also means that hereditary retinal diseases have, in terms of population genetics, only a very small negative selection effect, and this results in a rather high frequency of the alleles responsible for this group of diseases in the population. Another consequence of the fact that the disease is restricted to the retina is the impossibility to study tissue samples in an early stage of degeneration or to do meaningful biochemical studies on body fluids. Hence, investigation of these diseases is almost completely restricted to animal experiments.

A serious problem in studies of animals with a hereditary or experimentally induced retinal degeneration, is the lack of knowledge of the metabolic events in the normal retina, which renders interpretation of the results often difficult or impossible. Indeed,even fairly well investigated processes, e.g. the metabolism of rhodopsin and of its chromophoric group vitamin A, offer many unexplained phenomena. A continuous renewal and digestion of normal rod receptor outer segments has been demonstrated, but the source of *11-cis* retinaldehyde necessary for rhodopsin synthesis is not known. During the lifetime of a rhodopsin molecule (about 10 days in mammals) it is photolyzed and regenerated numerous times. During photolysis isomerization of *11-cis* to *all-trans* retinaldehyde takes place but localization and mode of re-isomerization to the *11-cis* configuration are not yet known. Investigations about the role of the pigment epithelium in this regeneration process have yielded contradictory results. Some investigators suggest that intact pigment epithelial function is absolutely necessary for these events. Others show evidence that regeneration is possible within the isolated retina. Apart from its role in the storage of vitamin A compounds and the fact that phagocytosis and digestion of rod outer segment lamellae takes place, the functions of the pigment epithelium in the complicated process

of photoreceptor metabolism have scarcely been investigated.

The processes just described are located exclusively in the retina, in contrast to many of the metabolic processes which take place in every living cell. Hence, for a better understanding of the errors of metabolism underlying hereditary retinal degenerations, first of all the processes which occur in the retina only, need to be investigated. Where possible the knowledge resulting from these investigations must be applied to the pathologic conditions, which for practical reasons must be done mostly in experimental animals.

CHAPTER 4

RHODOPSIN REGENERATION IN THE ISOLATED RAT RETINA

4.1 Introduction

Illumination of a vertebrate retina has a photochemical effect on the rhodopsin molecules in the rod outer segments, resulting in a chemical decomposition of the rhodopsin molecule, which initiates visual excitation. To retain visual sensitivity, rhodopsin must be resynthesised. This process is called regeneration. The way in which this regeneration takes place is still not understood. Upon illumination of rhodopsin, the chromophoric group *11-cis* retinaldehyde isomerizes to *all-trans* retinaldehyde, which is eventually released. For regeneration of rhodopsin almost certainly the decomposition products opsin and *all-trans* retinaldehyde recombine, resulting in a rhodopsin molecule. Hubbard and Wald (1952 a, b) showed that only *11-cis* retinaldehyde yields rhodopsin when incubated with opsin, so a re-isomerization from *all-trans* retinaldehyde to *11-cis* retinaldehyde is necessary for the regeneration process. It is not clear which conditions must be fulfilled in order to make possible isomerization from *all-trans* to *11-cis* vitamin A compounds. It is also not clear where re-isomerization takes place, in the rod outer segment or in the pigment epithelium, and whether it happens in the retinaldehyde or in the retinol form. It is an unique process that only occurs in the eye. Nowhere else in the body has the presence of *11-cis* vitamin A compounds been demonstrated.

In the living eye regeneration of rhodopsin can be easily observed (Rushton, 1952; Weale, 1953; Reuter, 1966; Dowling, 1960). Excised frog eyes regenerate part of their rhodopsin (Kühne, 1879; Dowling, 1962; Reuter, 1966). In isolated retinas the data on rhodopsin regeneration are contradictory. No regeneration of rhodopsin could be detected in isolated frog retinas (Goldstein, 1967; Crescitelli and Sickel, 1968) and isolated rat retinas (Goldstein, 1967). Even a perfused isolated rat retina with normal electric activity, as measured by ERG recordings, did not show any rhodopsin regeneration (Weinstein et al., 1967). On the other hand, homogenates of frog and rat retinas with pigmented layers

were able to regenerate rhodopsin in the presence of *all-trans* retinol (Collins et al., 1953). The possible importance of the pigment epithelium is also stressed by the observations of Ewald and Kühne (1878) and Hubbard and Wald (1951) that pigment epithelial material enhanced the rhodopsin regeneration in frog retina homogenates considerably.

The role of pigment epithelium in rhodopsin regeneration is denied by observations on rhodopsin regeneration in isolated rat retinas by Cone and Brown (1969). They claim that under special conditions regeneration in the isolated rat retina is possible. Isolated retinas of previously dark adapted albino rats were suspended in a Ringer solution in a very small chamber, only slightly larger in volume than the retina itself. The temperature of the chamber was maintained at 37°C. After bleaching a small area of 1 - 3 mmm diameter with 510-1000 nm light, a rhodopsin regeneration of 80% within 3 hours was recorded.
Their results are supported by Amer and Akhtar (1973 a, b). They gave evidence that partially bleached isolated rat and frog retinas incubated with tritiated *all-trans* retinaldehyde could convert *all-trans* retinaldehyde to *11-cis* retinaldehyde as indicated by thin layer chromatography. However, these authors did not present evidence that rhodopsin was actually formed.

It is clear that the pathways of vitamin A metabolism in the process of rhodopsin regeneration are still to a large extent unknown. The data on the role of the pigment epithelium in this process are contradictory. The site of re-isomerization of the visual pigment chromophore nor the chemical form during isomerization, whether alcohol or aldehyde, are known.

A simple system in which regeneration of rhodopsin takes place is offered by the experiments of Cone and Brown (1969). Because these experiments with isolated rat retinas seem a very promising model system of investigating the questions on rhodopsin regeneration just mentioned, attempts have been made to reproduce their results.

4.2 Rhodopsin regeneration in the isolated rat retina in an unperfused chamber

4.2.1 Materials and methods.

Albino Wistar rats are anesthetized with Nembutal (150 mg/kg)after dark adaptation for at least 12 hours. One eye is excised and the retina is dissected in dim red light (λ > 650 nm) in a drop of Ringer solution ($NaHCO_3$ 2mM, NaCl 125 mM, KCl 2mM, $CaCl_2$ 2mM, pH 6.8). The retina is then placed in a small chamber with a drop of the same Ringer solution and maintained at 37°C during the experiment. The chamber consists of two glass coverslips separated 0.4 mm by a polyvinylchloride ring with an inside diameter of 7 mm. The isolation procedure does not last more than 5 minutes. Absorption spectra are measured in a small area of $2mm^2$ in a split-beam recording spectrophotometer. In this instrument a measuring beam alternates between the retina and an opaque screen in balance with the light scattering qualities of the rat retina. One recording from 300 - 600 nm takes 30 seconds. A small area of 6 mm^2 is exposed to a flash of yellow light (λ > 510 nm) that bleaches about 80% of the rhodopsin present. Because the Ringer solution used by Cone and Brown appeared to maintain its pH very poorly, we have used in later experiments a buffer containing HEPES 10 mM, NaCl 115 mM, KCl 2 mM, $MgCl_2$ 2 mM, $CaCl_2$ 2 mM, glucose 10 mM (pH 6.8).

4.2.2 Results

In all experiments a decrease in absorption at 500 nm is observed after flash illumination, reflecting the disappearance of rhodopsin. In the next 10 minutes an increase and decrease at 380 nm and 470 nm indicates the transient appearance of metarhodopsin II and metarhodopsin III or free *all-trans* retinaldehyde. An increase at 340 nm in the following 15 minutes shows the formation of retinol. After the first half hour, no changes in absorption spectrum are recorded in the subsequent 4 hours. No rise in absorption at 500 nm is observed. Recorded spectra are shown in fig. 13. The first spectrum is recorded after placing the fully dark adapted retina in the experimental chamber. The retina is then flashed and successive spectra are recorded at the times shown in minutes after the flash.

4.2.3 Discussion

Cone and Brown (1969) stressed the fact that a small incubation volume and an illumination of only a part of the retina were crucial

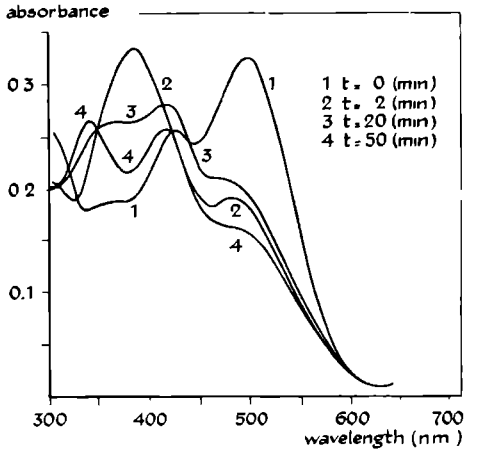

Fig. 13 Absorption spectra of the isolated rat retina at 37°C.

experimental factors, suggesting that one or more metabolic factors, involved in the rhodopsin regeneration and present in the retinal tissue, are subject to dilution in a larger volume of Ringer solution, and also that bleaching of the retina resulted in disappearance of the metabolic factor or factors.

However, although carefully observing the conditions employed by Cone and Brown (1969), no regeneration could be demonstrated in our experiments.

4.3 Rhodopsin regeneration in the isolated rat retina in a perfused chamber

4.3.1 Introduction

The results of our experiments with isolated rat retinas in an unperfused chamber are disappointing and at variance with the results of Cone and Brown (1969). Although it is clear that in our experiments regeneration does not take place, it has not been settled, which step in the regeneration process is missing. By adding different metabolites of the hypothetical regeneration cycle by means of a perfusing system to the retina, we have tried to study step by step the regeneration process.

Although Weinstein et al. (1967) could not demonstrate any regeneration of rhodopsin in the perfused rat retina, even though the retina was quite viable under the perfusion conditions as measured by ERG recordings, it could be that for the isomerization of *all-trans* to *11-cis* retinaldehyde and the subsequent formation of rhodopsin a substance is required which is washed out by the perfusion fluid. We have, therefore, added to the perfusion fluid various metabolites important for regeneration. At the same time we have kept the circulating volume as small as possible to minimize dilution of metabolites essential for the regeneration process.

It is known that retinas in opened excised frog eyes do regenerate in part their rhodopsin (Kühne, 1878; Hubbard and Dowling, 1962; Reuter, 1966). Because it might be that due to slight differences in the isolation procedure the isolated retina in the experiments of Cone and Brown (1969) was contaminated with pigment epithelial material, just enough to permit regeneration, we have also tested whether the addition of a suspension of pigment epithelium to the perfusate will result in regeneration of rhodopsin.

4.3.2 Materials and methods

Rat retinas are isolated as described under 4.2.1. After isolation they are placed in a stainless steel chamber, mounted on a piece of small gauze. The volume of the chamber itself is the same as that of the closed chamber described in section 4.2.1. The chamber can be maintained at a constant temperature of 37°C. Absorption spectra are measured through holes in the chamber covered with quartz slides in a split-beam recording spectrophotometer. Here the measuring beam alternates between the retina and an opaque screen in a chamber built exactly like that containing the retina, in balance with the light-scattering qualities of the rat retina. Both chambers are separately connected with a perfusion system, the total perfusing volume being not more than 4 ml. The flow of the perfusate is 1 ml per minute. The perfusate consists of a buffer solution containing HEPES 10 mM, NaCl 115 mM, KCl 2mM, $CaCl_2$ 2 mM, glucose 10 mM (pH 6.8). Vitamin A compounds, dissolved in a small amount of acetone, in later experiments in 2% digitonin solution, are added in a final concentration of 15 µM (40 fold molar excess towards retinal

rhodopsin) after bleaching. A small area of the retina is illuminated with a flash of yellow light ($\lambda > 510$ nm) which bleaches about 80% of the rhodopsin present. In one experiment to the perfusate is added a suspension of rat pigment epithelium from two pigmented rat eyes.

4.3.3 Results

Addition of *all-trans* retinol, *11-cis* retinol or *11-cis* retinaldehyde dissolved in a small volume of acetone has no effect at all on the absorption spectrum. Apparently the vitamin A compounds do not reach the chamber containing the retina in sufficient amounts, since we do not observe under these circumstances an increased light absorption in the near ultraviolet.

Addition of *all-trans* retinol, *11-cis* retinol or *11-cis* retinaldehyde dissolved in a small volume of 2% digitonin results in changes of the absorption spectrum, reflecting the presence of the vitamin A compounds in the chamber containing the retina, but still no regeneration of rhodopsin can be demonstrated.

Addition of a pigment epithelium suspension to the perfusate does not affect the absorption spectrum. No regeneration of rhodopsin occurs.

4.3.4 Discussion

The failure of the retina to regenerate rhodopsin, both when the conditions employed by Cone and Brown are carefully observed, and also after certain conditions are changed, is consistent with the negative results of Goldstein (1967) and Weinstein et al. (1967). Other factors, not mentioned by Cone and Brown (1969), probably influence the rhodopsin regeneration process. Many other experiments mentioned in the introduction of this chapter, in which rhodopsin regeneration occurred, have in common the presence of pigment epithelial tissue. So it is tempting to assume that the pigment epithelium plays a role in the regeneration process in these experiments. It could do so by offering essential metabolites, by inactivating substances inhibitory to the regeneration process, by changing the energy state of the visual pigment chromophore previous to an endergonic isomerization reaction in the photoreceptor, or by another unknown action.

The failure of the retina in the perfused chamber to regenerate rhodopsin after addition of a pigment epithelium suspension to the perfusate is not contradictory to the possibilities just mentioned, because the negative results in these experiments with a perfused retina seem largely the result of technical failure of reaching an interaction between substances added to the perfusate and the retina. This is proved by the fact that even addition of *11-cis* retinaldehyde to the perfusate does not result in any regeneration of rhodopsin, although bleached rat retinas, after a 3 hour incubation period in the perfusion chamber, when homogenized and incubated with a five-fold molar excess of *11-cis* retinaldehyde, yield a small but significant regeneration of rhodopsin.

PARTICULATE RETINOLDEHYDROGENASE OF ROD OUTER SEGMENT MEMBRANES FROM CATTLE AND RAT

5.1 Introduction

Retinoldehydrogenase is an important enzyme for the metabolism of vitamin A in the retina. It is needed for the conversion of vitamin A alcohol, which circulates in the blood, to vitamin A aldehyde, which is the form required as chromophoric group in the visual pigment rhodopsin. Thus it is the key in making the vitamin A pool of the body accessible to the visual system.

The presence of a membrane-bound retinoldehydrogenase has been reported by many authors for cattle rod outer segments (Wald 1949, 1950; Futterman and Saslaw, 1961; Futterman, 1963, 1965; Koen and Shaw, 1966; de Pont et al., 1970) and by some others for rat rod outer segments (Reading and Sorsby, 1966; Newhouse et al., 1972; Kissun et al., 1972). NADP is the preferred co-enzyme in cattle (Futterman, 1963; de Pont et al., 1970) as well as in rats (Graymore and Power, 1972; Newhouse et al., 1972; Graymore et al., 1974). The substrate, vitamin A, occurs in the eye predominantly in the retina and in the pigment epithelium. Apart from its storage form as retinylester, vitamin A is present in the aldehyde form and in the alcohol form.

The presence of two geometric configurations of these vitamin A compounds, *11-cis* and *all-trans*, makes the study of alcoholdehydrogenase function in the retina more complex. In this laboratory it has been shown that the retinoldehydrogenase bound to cattle rod outer segments cannot convert *11-cis* vitamin A compounds (Daemen et al., 1974). This means that either only *all-trans* vitamin A compounds are converted in the retina, or that other retinoldehydrogenases converting *11-cis* vitamin A compounds must be present elsewhere in the retina.

In view of this reported stereospecificity we have further characterized the stereospecific aspects of cattle rod outer segment bound retinoldehydrogenase, comparing these aspects with horse liver alcoholdehydrogenase under various circumstances. We have also investigated whether this phenomenon occurs more general in vertebrates by studying

the stereospecificity of retinoldehydrogenase in the normal rat retina as well. Finally we have studied the presence and the stereospecificity of a retinoldehydrogenase in pigment epithelium. The results of this study are reported in chapter 6.

5.2 Materials and methods

5.2.1 Substrates and co-enzymes

All-trans retinaldehyde is obtained from Eastman, Rochester, New York; *11-cis* retinaldehyde is prepared by photo-isomerization of an *all-trans* retinaldehyde solution in ethanol and elution of the *11-cis* isomer with benzene-hexane (1:9 v/v) from an aluminum oxide column (Brown and Wald, 1956). *All-trans* retinol and *11-cis* retinol are prepared by reducing the corresponding aldehyde with $NaBH_4$ in ethanol, followed by extraction with hexane. Experiments involving the vitamin A compounds are carried out in dim red light ($\lambda > 650$ nm) to prevent light-induced isomerization, and under nitrogen to prevent oxidation. NAD, NADP and the corresponding reduced products are obtained from Boehringer, Mannheim (Germany).

5.2.2 Enzyme sources

Unless otherwise indicated, bovine outer segment preparations are isolated by the method of de Grip et al. (1972) in daylight. After the first sucrose gradient of this procedure, a heavy sediment and two layers are obtained. The upper layer, which contains rod outer segments exclusively, is collected carefully to prevent contamination with the lower layer, and is diluted with an equal volume of Tris-HCl buffer (0.16 M, pH 7.1) and centrifuged for 15 minutes at 27,000 x g and $4^{\circ}C$. The sediment is washed twice with distilled water and sedimented after each washing (45,000 x g, $4^{\circ}C$, 30 minutes). After resuspension in an appropriate buffer the material is used as the enzyme preparation. During the normal isolation procedure in daylight all rhodopsin is bleached and the resulting retinol is nearly entirely washed out. When the isolation procedure is carried out in dim red light, the upper layer contains all rhodopsin.

Rod outer segment retinoldehydrogenase from rats is prepared from

albino Wistar strain animals, which are killed by cervical dislocation. The eyes are enucleated, the cornea lens and vitreous are removed, and the retinas are collected. The retinoldehydrogenase suspension is prepared by collecting and homogenizing the retinas in ice cold buffer solution (0.6 M Tris-HCl pH 7.2). The suspension is centrifuged for 20 min. at 20,000 x g and 4°C. The sediment is resuspended in 1 ml 0.1 M acetate buffer (pH 5.0).

Horse liver alcoholdehydrogenase which is purchased from Boehringer (Mannheim, Germany), contains 10% ethanol added as a preservative. Since ethanol interferes with the conversion of vitamin A compounds by this enzyme, the enzyme preparation is dialysed for 3 hours against the appropriate buffer (see below) shortly before it is used in an experiment.

5.2.3 Methods

Enzyme activity is determined by measuring the velocity of substrate conversion during the first 3 minutes of incubation. Both in the reduction and the oxidation reaction the amount of retinaldehyde present is measured as a function of incubation time, showing the disappearance of substrate or the appearance of product, respectively. Unless otherwise indicated, the reaction is carried out in 0.5 ml detergent-free medium, containing an amount of enzyme sufficient to give a conversion rate of maximally 20 nmol per 5 min. at 37°C. The substrate (60 nmol) is added in 10 µl methanol or peroxide-free dioxane. The presence of this small amount of organic solvents does not affect the enzyme activity. Normally the reduction reaction is carried out in 0.1 M Na-acetate buffer (pH 5.0) and the oxidation reaction in 0.1 M Tris-HCl buffer (pH 8.5). The reaction is started by adding 300 nmol of the appropriate cofactor, and at definite time intervals, aliquots are taken, frozen in dry ice/ethanol and later analyzed for their retinaldehyde content by the thiobarbituric acid method (Futterman and Saslaw, 1961; Daemen et al., 1970).

Product analysis is carried out by thin layer chromatography. The samples are repeatedly extracted with a double volume of hexane. The concentrated hexane solutions are applied to Silicagel 60 F 254 plates (Merck, Darmstadt, Germany) and eluted with 50% (v/v) ether-hexane in saturated chambers. The developed chromatograms are scanned with a Vitatron TLD-100 densitometer by measuring the absorption of transmitted light

from a tungsten light source provided with a filter, which transmits light of wavelengths between 250 and 400 nm.

With cattle rod outer segment retinoldehydrogenase and horse liver alcoholdehydrogenase, enzymatic formation of *11-cis* retinaldehyde is also tested by its reaction with opsin. The opsin preparation is the upper layer of the retina fractionation carried out in the light. The amount of rhodopsin formed during this reaction is determined by differential absorption spectrophotometry in a solution containing 1% Triton X-100 and 50 mM hydroxylamine in 0.1 M phosphate buffer (pH 7.0).

5.3 Results

5.3.1 Assay conditions

The retinoldehydrogenase located in rod outer segments is a membrane bound enzyme. Detergents have often been used in studies of this enzyme in view of its particulate nature and of the water insolubility of vitamin A compounds. However, the activity of bovine rod outer segment retinoldehydrogenase is seriously affected by detergents as shown in fig. 14.

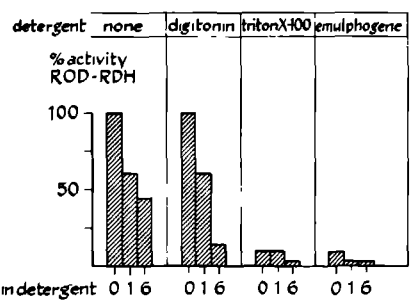

Fig. 14 Effect of various detergents on bovine rod outer segment retinoldehydrogenase activity.
Initial velocity of conversion of retinaldehyde is measured by incubating 0.5 ml enzyme suspension at 37°C with 60 nmol substrate in the presence of 300 nmol of NADPH in 0.1 M acetate buffer (pH 5.0) with or without 1% (w/v) detergent. The assay is repeated after keeping the enzyme suspensions for 1 and 6 hours at 20°C.

Therefore, we have used suspensions of particulate material without detergent and have added the substrate in a minimal amount of organic solvent. Unfortunately, liver alcoholdehydrogenase shows no activity at all towards retinaldehyde and retinol, unless a detergent or membrane material, e.g. enzymatically inactive opsin, is added. Therefore, all experiments with liver alcohol dehydrogenase have been performed in 0.1% Triton X-100, in which the enzyme is stable and active.

The effective substrate concentration of vitamin A compounds in a membrane suspension is difficult to assess, since the substrate accumulates in the lipid phase of the membrane material. This precludes a realistic interpretation of binding and catalytic properties in terms of K_m and V_{max} values. The results of our experiments are therefore presented on a strictly comparative basis for substrate conversion rates of about 20 nmol per 5 min. at 37°C.

5.3.2 Optimal pH of the bovine retinoldehydrogenase and the horse liver alcoholdehydrogenase

The optimal pH for both retinaldehyde reduction and retinol oxidation are determined by measuring initial velocities of substrate conversion over a pH-range of 4.5 to 9.0, using *all-trans* vitamin A compounds as substrate. The results are depicted in figs. 15 and 16.

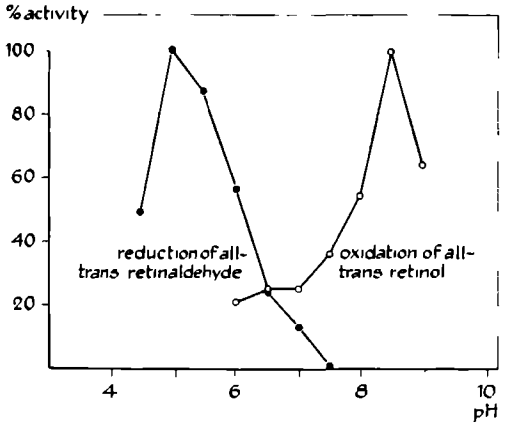

Fig. 15 Dependence on pH of bovine rod retinoldehydrogenase activity.

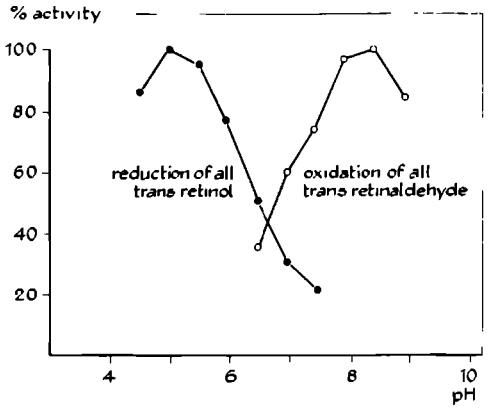

Fig. 16 Dependence on pH of horse liver alcoholdehydrogenase activity

5.3.3 Cofactor specificity of the bovine enzyme and horse liver enzyme

The data on cofactor specificity are presented in table I. The relative co-enzyme preference is expressed as the ratio of the initial velocity of the reaction with NADP(H) to that with NAD(H), using *all-trans* vitamin A compounds as substrate.

Table I

	Ratio initial velocity NADP/NAD	
	reduction	oxidation
Bovine retinoldehydrogenase	1.5	3.0
Horse liver alcoholdehydrogenase	0.6	0.5

The bovine rod retinoldehydrogenase clearly shows preference for NADP, while horse liver alcoholdehydrogenase does show a preference for NAD. In all subsequent experiments we have used NADP as co-enzyme for the rod retinoldehydrogenases and NAD as co-enzyme for horse liver alcoholdehydrogenase.

5.3.4 Stereospecificity of the bovine enzyme

The stereospecificity is tested by comparing the kinetics of substrate conversion and by analyzing the products of enzymatic conversion by means of thin layer chromatography. The latter approach is described in the next section (5.3.5).

The conversion rates of the *all-trans* and *11-cis* vitamin A compounds by bovine rod retinoldehydrogenase and horse liver alcoholdehydrogenase have been compared under the optimal conditions described before. The results are depicted in fig. 17 and fig. 18.

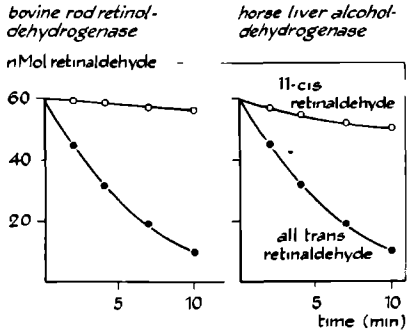

Fig. 17 Stereospecificity of bovine rod retinoldehydrogenase and horse liver alcoholdehydrogenase in the reduction of retinaldehyde.
The activities are measured by incubating 60 nmol substrate with a fresh enzyme preparation in 0.1 M acetate buffer (pH 5.0) at 37°C, containing 300 nmol of co-enzyme. In the case of the retinoldehydrogenase the co-enzyme is NADPH, while in the case of the alcoholdehydrogenase it is NADH.

It is clearly shown that cattle rod outer segment retinoldehydrogenase converts *11-cis* vitamin A compounds very slowly, presumably only after previous isomerization to the *all-trans* form, whereas horse liver alcoholdehydrogenase, although showing a preference for *all-trans* vitamin A compounds, converts *11-cis* vitamin A compounds to a considerable extent.

The same experiments carried out at a physiological pH (7.0) show

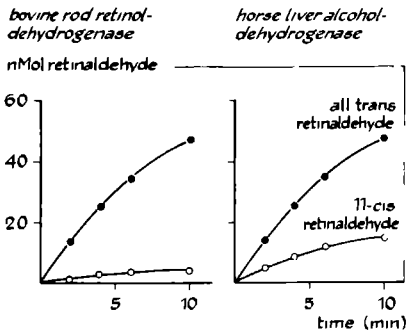

Fig. 18 Stereospecificity of bovine rod retinoldehydrogenase and horse liver alcoholdehydrogenase in the oxidation of retinol.
The activities are measured by incubating 60 nmol substrate with a fresh enzyme preparation in 0.1 M Tris-HCl buffer (pH 8.5) at 37°C, containing 300 nmol of co-enzyme. In the case of the retinoldehydrogenase the co-enzyme is NADP, while in the case of the alcohol-dehydrogenase is in NAD.

about the same specificity towards the stereo isomers of retinaldehyde and retinol.

5.3.5 Product analysis by thin layer chromatography

The *11-cis* vitamin A compounds are subject to aspecific isomerization under the experimental conditions. This means that the slight conversion of *11-cis* vitamin A by the bovine rod enzyme may be an apparent one only, due to isomerization of the substrate prior to conversion. Therefore it is important to analyze substrates and products during the enzymatic conversion reaction. Thin layer chromatography permits separation of the various isomers of retinaldehyde and retinol. The results of this analysis are shown in fig. 19 and fig. 20.
On the right the positions of the retinaldehyde and retinol isomers on the developed chromatogram are shown. The curves represent densitometric scans of developed chromatograms from the hexane-extracted vitamin A

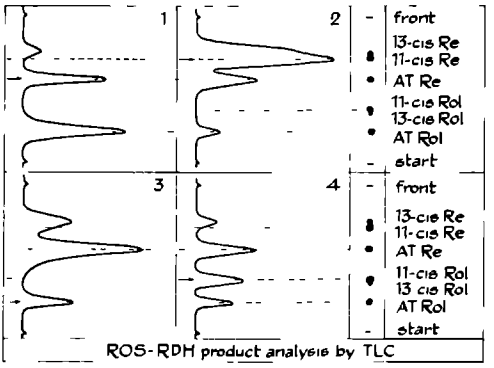

Fig. 19 Bovine rod outer segment retinoldehydrogenase: product analysis by thin layer chromatography. Re = retinaldehyde; Rol = retinol
Arrow indicates substrate in each case
Substrates: 1. *All-trans* retinaldehyde
2. *11-cis* retinaldehyde
3. *All-trans* retinol
4. *11-cis* retinol

compounds after incubation with the enzyme preparation and the appropriate cofactor for 30 minutes at 37°C under nitrogen, the amounts of substrate, enzyme and cofactor being the same as in the kinetic experiments. These patterns show that in the case of bovine rod retinoldehydrogenase no *11-cis* vitamin A compounds are formed as a product of *11-cis* vitamin A substrates, while in the case of horse liver alcoholdehydrogenase *11-cis* vitamin A compounds are converted to *11-cis* products, although to a lesser extent than *all-trans* vitamin A compounds. It is also apparent that in the presence of this water soluble phospholipid-free enzyme almost no aspecific isomerization takes place, whereas in the case of incubation with rod outer segment membranes substantial aspecific isomerization takes place.

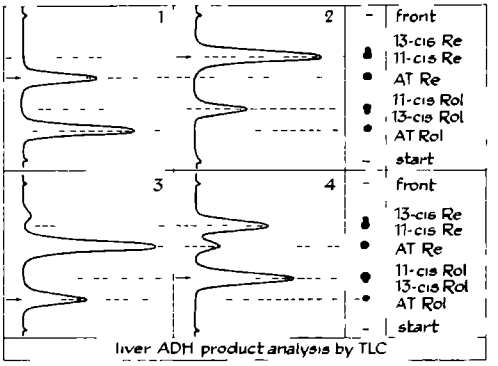

Fig. 20 Horse liver alcoholdehydrogenase: product analysis by thin layer chromatography
Re = retinaldehyde; Rol = retinol
Arrow indicates substrate in each case.
Substrates: 1. *All-trans* retinaldehyde
2. *11-cis* retinaldehyde
3. *All-trans* retinol
4. *11-cis* retinol

5.3.6 Detection of the formation of *11-cis* retinaldehyde by its ability to form rhodopsin from opsin

A very specific and conclusive way to detect formation of *11-cis* retinaldehyde is its reaction with opsin to form rhodopsin. The experimental conditions are as follows: 15 nmol cattle opsin with retinoldehydrogenase activity is incubated for 30 min. in the dark at 37°C with 75 nmol *11-cis* retinol and 350 nmol $NADP^+$ in 1 ml 0.1 M phosphate buffer (pH 7.0). In the next experiment horse liver alcoholdehydrogenase is present in the incubation mixture. In control experiments opsin alone is incubated, and opsin is incubated with 40 nmol *11-cis* retinaldehyde. The results are collected in table II. Fig. 21 shows the absorption

spectrum of the reaction products.

Table II Enzymatic oxidation of *11-cis* retinol to *11-cis* retinaldehyde measured by rhodopsin formation.

Incubation mixture	A_{500}
Opsin (= bovine rod retinoldehydrogenase)	0.010
Opsin + *11-cis* retinol + $NADP^+$	0.040
Opsin + *11-cis* retinol + NAD^+ + horse liver alcoholdehydrogenase	0.460
Opsin + *11-cis* retinaldehyde	0.440

Table II proves that no formation is seen, when opsin with retinoldehydrogenase activity (tested by kinetic experiments) is incubated with *11-cis* retinol in the presence of $NADP^+$. However, addition of horse liver alcoholdehydrogenase to this system results in the conversion of all opsin to rhodopsin in the presence of NAD^+. Fig. 21 shows that the

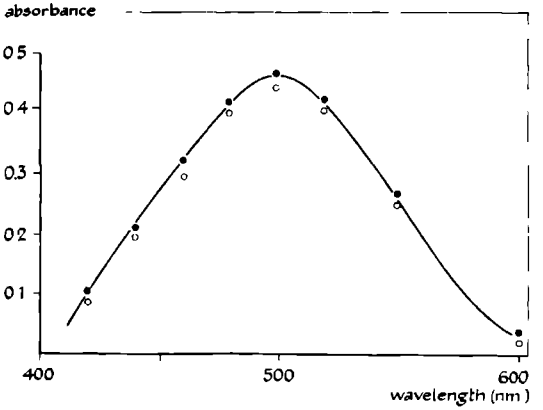

Fig. 21 Spectrum of the product resulting from reacting opsin and enzymatically oxidized *11-cis* retinol.
● Horse liver alcoholdehydrogenase + *11-cis* retinol + NAD^+
○ Opsin + *11-cis* retinaldehyde (control)

product resulting from reacting opsin and enzymatically oxidized *11-cis* retinol is indeed rhodopsin, with an absorption maximum at 500 nm.

5.3.7 Stereospecificity of the rat enzyme

We have also investigated the stereospecificity of rod outer segment retinoldehydrogenase in the rat, in order to assess whether the same enzyme is present in other vertebrates. Because isolation of rod outer segments from rat retinas turns out to be rather difficult and results in a substantial loss of activity, we have used whole retina homogenates.

5.3.8 Stereospecificity of conversion of vitamin A compounds by rod outer segment retinoldehydrogenase

The kinetics of the retinaldehyde reduction show a clear preference for *all-trans* retinaldehyde, the ratio of conversion of *11-cis* retinaldehyde versus *all-trans* retinaldehyde being about 0.3.

Product analysis by thin layer chromatography indicates that both in the reduction and oxidation of *11-cis* vitamin A compounds no *11-cis* products are formed, but only *all-trans* products. The conversion is considerably less than when *all-trans* vitamin A compounds are subjected to enzymatic conversion, thus showing again a high stereospecificity towards *all-trans* vitamin A compounds.

5.4 Discussion

The presence of *11-cis* vitamin A compounds in the eye is of great importance for visual function. Because this geometric configuration is rather unstable, only a small amount of energy is required for its isomerization. The isomerization itself causes a large conformatial change, leading to changes in the rod sac membrane and ultimately to visual excitation. Only in the *11-cis* configuration do vitamin A molecules show these large effects after absorption of photic energy. The fact that an extra-ocular enzyme system like liver alcoholdehydrogenase shows little stereospecificity towards the vitamin A isomers, stresses the importance of the high stereospecificity of the bovine rod outer segment bound

retinoldehydrogenase. Experiments with rat retinas indicate that this stereospecificity may be present in all vertebrates, including man. These experiments, carried out in whole retina homogenates, also indicate that no other retinoldehydrogenase is present in the retina of the rat which is able to convert *11-cis* vitamin A compounds.

The role of the *all-trans* specific retinoldehydrogenase, closely attached to the rhodopsin containing membranes of the rod outer segment cannot be to catalyze the oxidation of *11-cis* retinol to *11-cis* retinaldehyde for visual pigment regeneration. This suggests that *all-trans* retinaldehyde, liberated on bleaching of rhodopsin, can be isomerized to *11-cis* retinaldehyde in or near the rod outer segment disc membranes. If the *all-trans* retinaldehyde would first be reduced before isomerization, the resulting *11-cis* retinol cannot be converted to *11-cis* retinaldehyde required for rhodopsin regeneration.

The classic rhodopsin cycle according to Wald (1968) shown in fig. 10 thus needs some alteration. In its place a simplified rhodopsin cycle located in the rod outer segment may then be proposed (Daemen et al., 1974), shown in fig. 22.

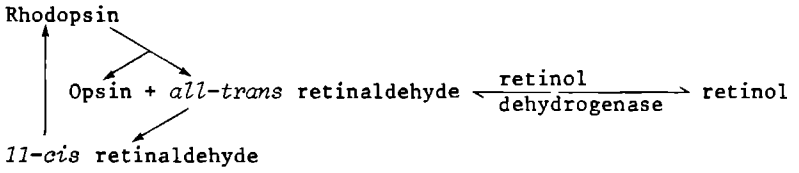

Fig. 22 Simplified rhodopsin cycle, located in the rod outer segment

It is improbable that *11-cis* retinaldehyde as such moves from another localization, e.g. the pigment epithelium, to the rod outer segment because the very reactive aldehyde group will prevent the molecule from migration. Since our results indicate that all the *11-cis* retinaldehyde required for visual pigment regeneration is produced by isomerization of *all-trans* retinaldehyde, it would be the function of the rod outer segment retinoldehydrogenase to provide *all-trans* retinaldehyde from a source of *all-trans* retinol, or to convert *all-trans* retinaldehyde to

all-trans retinol. By these actions it could regulate the amount of retinaldehyde present in the rod outer segment, according to the state of dark adaptation, and connect the retinol pool in the pigment epithelium to the photoreceptor metabolism.

CHAPTER 6

PARTICULATE RETINOLDEHYDROGENASE OF THE RETINAL PIGMENT EPITHELIUM FROM CATTLE AND RAT

6.1 Introduction

The pigment epithelium, which originates from the same embryological ependym layer as the retinal photoreceptor layer and is in close contact with the latter in the adult eye, forms a functional unit with the photoreceptor cells. The nourishment of the photoreceptor layer is dependent on the pigment epithelium, which on its opposite side is in contact with the choriocapillaris (Moyer, 1969). In mammals about 10% of the rod outer segment membranes is daily engulfed and degraded by the pigment epithelium as part of the continuous rod renewal cycle (Young and Bok, 1969; Spitznas and Hogan, 1970). In cold blooded animals like frog about 2% is daily turned over. During light and dark adaptation a constant shift of vitamin A compounds to and from the pigment epithelium takes place (Dowling, 1960; Zimmerman, 1974). The pigment epithelium acts as an important depot for retinol in an esterified form (Krinsky, 1958; Berman et al., 1974).

Many steps of retinal vitamin A metabolism, which is of crucial importance for photoreceptor function, obviously take place in the pigment epithelium. Kissun et al. (1972) provided histochemical evidence for the presence of retinoldehydrogenase activity in the pigment epithelium of the rat. We have investigated enzymic activity towards vitamin A compounds in cattle and rat pigment epithelium, with special attention to its substrate specificity.

6.2 Materials and methods

6.2.1 Enzyme source

Cattle pigment epithelium is isolated by gently scraping it off the choroidal tissue of fresh bovine eyes from which the retina has previously been removed carefully. This material is homogenized in ice-cold Tris-HCl buffer (0.16 M, pH 7.1) and centrifuged for 5 minutes at

500 x g and 4°C. The supernatant is then centrifuged for 1 hour and 43,000 x g and 4°C. The resulting sediment is washed twice with distilled water. After resuspending it in the appropriate buffer, the material is used as the enzyme preparation. When the procedure is carried out in dim red light, the preparation contains no rhodopsin, indicating that it is free of rod outer segments.

For isolation of pigment epithelial retinoldehydrogenase from rats experimental animals of the albino Wistar strain are killed by cervical dislocation, the eyes are enucleated and after removal of cornea, lens, vitreous and retina, the remaining eye cups, containing the pigment epithelium are collected in ice-cold 0.25 M sucrose - 1 mM EDTA - 0.01 M Tris-HCl (pH 7.8) and homogenized with a Polytron homogenizer for 10 seconds. The homogenate is centrifuged for 5 minutes at 10,000 x g and 4°C. The sediment is again homogenized the same way and after centrifugation the collected supernatants are centrifuged for 1 hour at 100,000 x g and 4°C. The resulting sediment is washed two times with 0.1 M acetate buffer (pH 5.0) and ultimately suspended in 0.1 M acetate buffer (pH 5.0).

6.2.2 Substrates and coenzymes

All-trans retinaldehyde is obtained from Eastman, Rochester, New York; *11-cis* retinaldehyde is prepared by photo-isomerization of an *all-trans* retinaldehyde solution in ethanol and elution of the *11-cis* isomer with benzene-hexane (1:9 v/v) from an aluminum oxide column (Brown and Wald, 1956). *All-trans* retinol and *11-cis* retinol are prepared by reducing the corresponding aldehyde with $NaBH_4$ in ethanol, followed by extraction with hexane. Experiments involving the vitamin A compounds are carried out in dim red light ($\lambda > 650$ nm) to prevent light-induced isomerization, and under nitrogen to prevent oxidation.
NAD is obtained from Boehringer, Mannheim, Germany, as are NADP and the corresponding reduced products.

6.2.3 Enzyme assay

Enzyme activity is determined by measuring the velocity of substrate conversion during the first 3 minutes of incubation. Both in the reduction reaction and in the oxidation reaction the amount of retinaldehyde

present is measured as a function of incubation time. This method is described in more detail in chapter 5, section 2.3.

6.2.4 Product analysis

The products of enzymatic conversion are analysed by thin layer chromatography, carried out on Silica Gel with 50% (v/v) ether-hexane as eluent. More details on this method are described in chapter 5, section 2.3.

With cattle pigment epithelium retinoldehydrogenase enzymatic formation of *11-cis* retinaldehyde is also tested by its reaction with opsin. The opsin preparation is the upper layer of the retina fractionation described in chapter 5 section 2.2, carried out in the light. The amount of rhodopsin formed during this reaction is determined by differential absorption spectrophotometry in a solution containing 1% Triton X-100 and 50 mM hydroxylamine in 0.1 M phosphate buffer (pH 7.0).

6.3 Results

6.3.1 Detection of the enzyme in cattle pigment epithelium

In preliminary experiments scraped off pigment epithelial material homogenate has been used without further purification. Enzyme activity is tested by kinetic experiments and by product analysis on thin layer chromatography.

The results of the kinetic experiments are depicted in fig. 23. Cattle pigment epithelium homogenate catalyzes the conversion of *11-cis* retinol to retinaldehyde and to a lesser extent the conversion of *all-trans* retinol to retinaldehyde. NAD^+ is an efficient cofactor in this reaction.

Analysis of the products of enzymatic conversion by thin layer chromatography shows that part of the products of *11-cis* retinol conversion is *11-cis* retinaldehyde. *13-cis* retinaldehyde and *all-trans* retinaldehyde are also present, probably due to aspecific isomerization. For the same reason the substrate *11-cis* retinol is partly isomerized to *all-trans* retinol. Heating makes the homogenate devoid of activity.

From these data we can conclude that crude pigment epithelium homogenate contains a retinoldehydrogenase activity with preference for *11-*

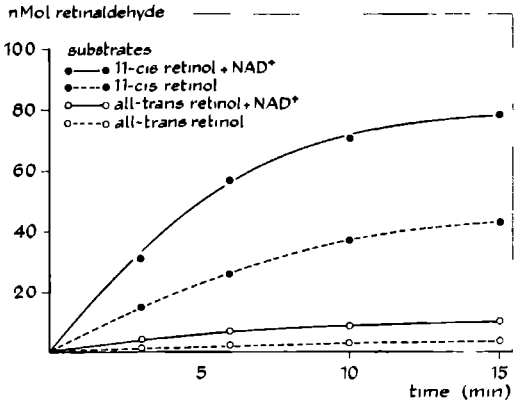

Fig. 23 Stereospecificity and co-enzyme dependency of pigment epithelial retinoldehydrogenase.
0.4 µmol *11-cis* retinol or *all-trans* retinol in 10 µl methanol is added to 1 ml pigment epithelium homogenate in 0.16 M Tris-HCl buffer (pH 7.1) and incubated at 37°C with or without addition of 2 umol NAD^+. Aliquots are assayed for retinaldehyde content.

cis retinol, which can convert *11-cis* retinol to *11-cis* retinaldehyde and, to a lesser extent, *all-trans* retinol to *all-trans* retinaldehyde.

We have further characterized this enzyme by simple purification and by investigating its functional parameters.

6.3.2 Purification of the bovine enzyme

Pigment epithelial material of fresh cattle eyes is isolated in daylight as described in section 6.2 and homogenized in 0.16 M Tris-HCl buffer (pH 7.1). Samples of this homogenate are centrifuged for different times and different speeds at 4°C. Samples of the resulting sediments, resuspended in the same buffer solution, and of the supernatants are assayed for retinoldehydrogenase activity by assaying the conversion of *11-cis* retinol to retinaldehyde by the thiobarbituric acid method. The results are summarized in Table III. Centrifugation for 5 minutes at 500 x g sediments coarse cell debris and results only in a slight loss of enzyme activity. Centrifugation for 30 minutes at 43,000 x g sediments most of the enzyme material. Therefore it seems probable that the enzyme

Table III

Centrifugation time	Gravitational force	% of total activity in sediment
5 minutes	500 x g	15%
30 minutes	43,000 x g	60%
60 minutes	200,000 x g	70%
15 hours	200,000 x g	100%

has a particulate character.

On the basis of these results we have chosen for the enzyme isolation method further used in this chapter. More experimental details on the localisation of the enzyme in the pigment epithelium are given in chapter 7.

6.3.3 Assay conditions

Although cattle rod outer segment retinoldehydrogenases activity is seriously affected by detergents (chapter 5, section 3.1) cattle pigment epithelium retinoldehydrogenase activity is much less susceptible to inactivation by detergents as shown in fig. 24. Sometimes even an activation of the enzyme activity is found, but this is a rather inconstant finding. To be able to compare the ocular retinoldehydrogenase we have used suspensions of the enzyme material without detergents.

Because the pigment epithelium retinoldehydrogenase has a particulate character, the effective substrate concentration of vitamin A compounds in the suspension is difficult to assess, since the substrate accumulates in the lipid phase of the material. This precludes a realistic interpretation of binding and catalytic properties in terms of K_m and V_{max} values. The results of the enzyme experiments are therefore presented on a strictly comparative basis for substrate conversion rates of about 20 nmol per 5 min. at 37°C.

6.3.4 Optimal pH of the bovine enzyme

The optimal pH for both retinaldehyde reduction and retinol oxidation are determined by measuring initial velocities of substrate conver-

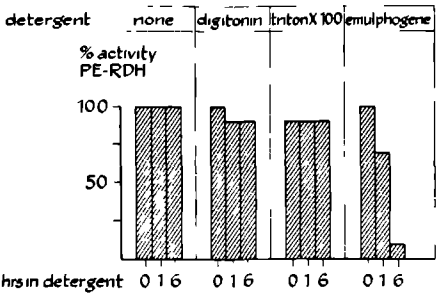

Fig. 24 Effect of various detergents on bovine pigment epithelium retinoldehydrogenase.
Initial velocity of conversion of *11-cis* retinaldehyde is measured by incubating 0.5 ml enzyme suspension at 37°C with 60 nmol substrate in the presence of 300 nmol of NADH in 0.1 M acetate buffer (pH 5.0) with or without 1% (w/v) detergent. The assay is repeated after keeping the enzyme suspensions for 1 and 6 hours at 20°C.

sion over a pH-range of 4.5 to 9.0 using *11-cis* vitamin A compounds as substrate. The results are depicted in fig. 25. The optimal pH for reduction is 5.0, for oxidation 8.5.

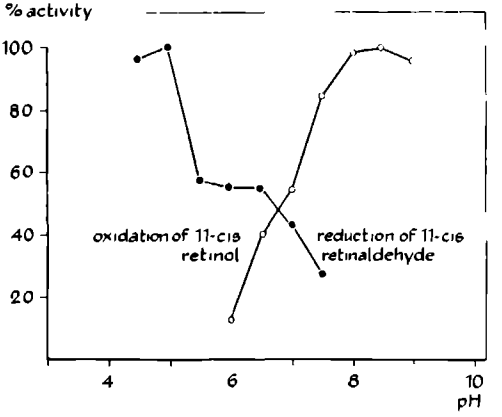

Fig. 25 Dependence on pH of bovine pigment epithelium retinoldehydrogenase activity.
●——● reduction of *11-cis* retinaldehyde
○——○ oxidation of *11-cis* retinol

6.3.5 Cofactor specificity of the bovine enzyme

The data on cofactor preference are presented in table IV. The relative co-enzyme preference is expressed as the ratio of the initial velocity of the reaction with NADP(H) to that with NAD(H), using *11-cis* vitamin A compounds as substrate. For comparison the ratios of the bovine rod outer segment retinoldehydrogenase are also given.

Table IV

	Ratio initial velocity NADP/NAD	
	reduction	oxidation
Bovine pigment epithelium retinoldehydrogenase	1.0	0.6
Bovine rod outer segment retinoldehydrogenase	1.5	3.0

In contrast with bovine rod outer segment retinoldehydrogenase, the bovine pigment epithelium retinoldehydrogenase does not show a clear co-enzyme preference. In all subsequent experiments we have used NAD as co-enzyme for the pigment epithelium retinoldehydrogenases.

6.3.6 Stereospecificity of the bovine enzyme

The stereospecificity is tested by comparing the kinetics of substrate conversion and by analyzing the products of enzymatic conversion by means of thin layer chromatography. The latter approach is described in the next section (6.5.6).

The conversion rates of the *all-trans* and *11-cis* vitamin A compounds have been compared under the optimal conditions described before. The results are depicted in fig. 26.
The bovine pigment epithelial enzyme clearly prefers *11-cis* vitamin A compounds, in contrast with the rod outer segment enzyme. However, the pigment epithelium enzyme is able to convert *all-trans* vitamin A compounds to a certain extent.

The same experiments carried out at a physiological pH (7.0) show about the same specificity towards the stereo-isomers of retinaldehyde and retinol.

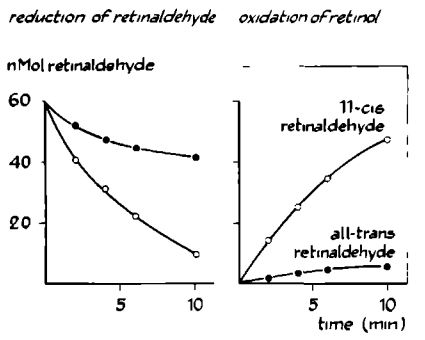

Fig. 26 Stereospecificity of bovine pigment epithelium retinoldehydrogenase in the conversion of vitamin A compounds.
The activities are measured by incubating 60 nmol substrate with a fresh enzyme preparation in the appropriate buffer at 37°C, containing 300 nmol of the appropriate co-enzyme.

6.3.7 Product analysis by thin layer chromatography

Although kinetic experiments with bovine pigment epithelium retinoldehydrogenase show a clear preference for *11-cis* vitamin A compounds, these experiments alone do not prove that the products of this enzymatic conversion have still the same geometric configuration as the substrate. Therefore, and also to obtain information about aspecific isomerization under the experimental conditions, it is important to analyze substrates and products during the enzymatic conversion reaction. Thin layer chromatography permits separation of the various isomers of retinaldehyde and retinol. The results of this analysis are shown in fig. 27. On the right the positions of the retinaldehyde and retinol isomers on the developed chromatogram are shown. The curves represent densitometric scans of developed chromatograms from the hexane-extracted vitamin A compounds after incubation with the enzyme preparation and the appropriate cofactor for 30 minutes at 37°C under nitrogen, the amounts of substrate, enzyme and cofactor being the same as in the kinetic experiments. These patterns show that the bovine pigment epithelium retinoldehydrogenase is indeed

capable of converting *11-cis* substrates to *11-cis* products, although substantial aspecific isomerization takes place.

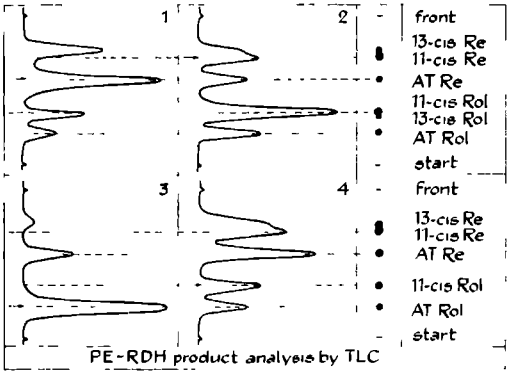

Fig. 27 Bovine pigment epithelium retinoldehydrogenase: product analysis by thin layer chromatography.
Re = retinaldehyde; Rol = retinol.
Arrow indicates substrate in each case.
Substrates: 1. *All-trans* retinaldehyde
2. *11-cis* retinaldehyde
3. *All-trans* retinol
4. *11-cis* retinol

6.3.8 Detection of the formation of *11-cis* retinaldehyde by its ability to form rhodopsin from opsin

In chapter 5 section 3.6 we found that opsin with retinoldehydrogenase activity was not able to convert *11-cis* retinol to *11-cis* retinaldehyde because no rhodopsin was formed. We repeated this experiment with bovine pigment epithelium retinoldehydrogenase. The experimental conditions are as follows: 15 nmol cattle opsin is incubated for 30 min. in the dark at 37°C with 75 nmol *11-cis* retinol and 350 nmol $NADP^+$ in 1 ml 0.1 M phosphate buffer (pH 7.0) in the presence of pigment epithelium

enzyme. The following control experiments have been included: one in which the pigment epithelium enzyme is omitted, one in which opsin alone is incubated, and one in which opsin is incubated with 40 nmol *11-cis* retinaldehyde. The results are collected in table V. Fig. 28 shows the absorption spectra of the reaction products.

Table V Enzymatic oxidation of *11-cis* retinol to *11-cis* retinaldehyde measured by rhodopsin formation.

Incubation mixture	A_{500}
Opsin	0.010
Opsin + *11-cis* retinol + NAD^+	0.040
Opsin + *11-cis* retinol + NAD^+ + bovine pigment epithelium retinoldehydrogenase	0.460
Opsin + *11-cis* retinaldehyde	0.440

Table V proves that in the presence of the pigment epithelium enzyme all opsin is converted to rhodopsin when *11-cis* retinol and NAD^+ is present in the incubation mixture. Fig. 28 shows that the product resulting from reacting opsin and enzymatically oxidized *11-cis* retinol is indeed rhodopsin, with an absorption maximum at 500 nm.

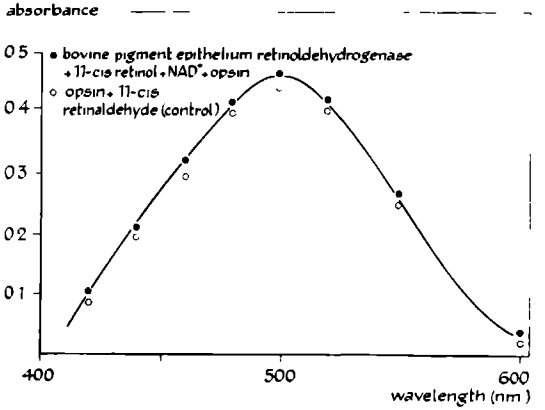

Fig. 28 Spectrum of the product resulting from reacting opsin and enzymatically oxidized *11-cis* retinol.
● Bovine pigment epithelium retinoldehydrogenase + *11-cis* retinol + NAD^+ + opsin.
○ Opsin + *11-cis* retinaldehyde (control)

6.3.9 Stereospecificity of the rat pigment epithelial enzyme

We have also investigated the stereospecificity of pigment epithelium retinoldehydrogenase in the rat, in order to assess whether the same enzyme is present in different vertebrates. One difference is immediately apparent: in our experiments with the rat enzyme, no measurable activity towards the retinols is found, although the activity towards the retinaldehydes can be measured quite well. In cattle pigment epithelium the reduction activity is only a factor 2 higher than the oxidation activity. In the rat this difference is presumably much greater. Since we consider only the reduction activity as physiologically important (see Discussion 6.4) we have not further investigated the oxidation activity of the rat enzyme.

The kinetics of the retinaldehyde reduction show a clear preference for *11-cis* retinaldehyde, the ratio of conversion of *11-cis* retinaldehyde versus *all-trans* retinaldehyde being about 7.0.

Product analysis by thin layer chromatography indicates that in the reduction of *11-cis* retinaldehyde, *11-cis* retinol is a product. The conversion is considerably less when *all-trans* retinaldehyde is subjected to enzymatic conversion, thus indicating again a preference towards *11-cis* retinaldehyde.

6.4 Discussion

The enzyme discussed in this chapter has not been characterized before. Histologic evidence for the presence of a retinoldehydrogenase in rat pigment epithelium has been given by Kissun et al. (1972). We have shown the presence of a retinoldehydrogenase in pigment epithelium with properties remarkably different from the retinoldehydrogenase present in rod outer segments. It is present both in cattle and in the rat, which makes it probable, that the same enzyme is present in humans as well.

We have various reasons to believe that we are dealing with an enzyme different from rod outer segment retinoldehydrogenase. The isolation procedure largely separates the retinal tissue from the pigment epithelium. The first centrifugation step during the isolation procedure sediments all rod outer segment material. The supernatant contains no rhodopsin, when the isolation is carried out in the dark. The difference

in behaviour towards detergents, the different cofactor preference, and, most importantly, the different stereospecificity towards *11-cis* and *all-trans* vitamin A compounds, are proof that we are dealing with at least two different retinoldehydrogenases in the eye.

What may be the role of the two different retinoldehydrogenases in the eye, especially with regard to their stereospecificity? The possible consequences of the stereospecificity of the rod outer segment enzyme have been discussed in chapter 5. In short, since *11-cis* retinaldehyde cannot be a product of conversion of retinol by this enzyme, re-isomerization to the *11-cis* configuration, necessary for visual pigment regeneration in the rod outer segment, should take place directly from *all-trans* retinaldehyde, unless another source of *11-cis* retinaldehyde is available for the rod outer segments. The suggestion that the pigment epithelium might serve as such a source is improbable.

First, although stores of *11-cis* retinylesters have been shown to exist in cattle and frog pigment epithelium (Krinsky, 1958; Hubbard and Dowling, 1962), these *11-cis* compounds are not preferentially used for rhodopsin regeneration during dark adaptation (Hubbard and Dowling, 1962) as compared with the esters of *all-trans* retinol. Secondly, transport of retinaldehyde has never been observed, either in the eye (Dowling, 1960; Zimmerman, 1974) nor elsewhere in the body, and is unlikely in view of the reactivity of the aldehyde group. Finally, free *11-cis* retinaldehyde is susceptible to nonspecific (i.e. light and enzyme independent) isomerization to other geometric configurations (Daemen et al., 1974; Futterman and Futterman, 1974). Thus, in spite of the fact that the pigment epithelium contains enzymes able to catalyze the conversion of *11-cis* retinylesters, via *11-cis* retinol (Krinsky, 1958), to *11-cis* retinaldehyde, it is improbable that the pigment epithelium acts as a source of *11-cis* retinaldehyde for regeneration. It is therefore unlikely that the outer segment obtains *11-cis* retinaldehyde as such from the pigment epithelium. A specific transport protein for *11-cis* retinaldehyde would have to be involved, and no evidence of such a protein has been found so far.

If the *11-cis* retinaldehyde is formed inside the outer segment, and *11-cis* retinol cannot be oxidized by the outer segment retinoldehydrogenase,its immediate precursor, at least in cattle, must be *all-trans*

retinaldehyde. This could be *all-trans* retinaldehyde, either that released during photolysis of rhodopsin or that formed by the oxidation of *all-trans* retinol derived from the pigment epithelium (Zimmerman et al., 1974).

This leaves open the function of the *11-cis* specific retinoldehydrogenase of the pigment epithelium. There is another source of *11-cis* vitamin A compounds in the pigment epithelium: rhodopsin, which is digested after apical packages of rod discs have been scavenged by the pigment epithelium. This process has been described in detail by Young and Bok (1969). In mammals every day about 1/10 of the rhodopsin content of the eye is taken up by the pigment epithelium and digested. Indeed Shichi (1973) has provided direct spectrophotometric evidence that rhodopsin and what is probably a product of its partial degradation occur in cattle pigment epithelium. It is possible that the *11-cis* retinol dehydrogenase we have found in the pigment epithelium is involved in this process and reduces the chromophoric group, apparently still in the *11-cis* configuration, to retinol. This in turn would be esterified to retinyl esters by an esterase which in cattle has been shown to exhibit no geometric preference towards *all-trans* and *11-cis* retinol (Krinsky, 1958). This hypothesis is supported by the observations of Hubbard and Dowling (1962) that in the frog the percentage of *11-cis* isomer in the pigment epithelium pool of retinylesters slowly increases over a period of 24 hours or more in continuous darkness. The geometric stability of *11-cis* retinyl esters, as opposed to *11-cis* retinaldehyde and *11-cis* retinol (Daemen et al., 1974) might be related to the completely hydrophobic conditions in the lipid droplets, which are believed to contain the retinyl esters in the pigment epithelium. This store of retinyl esters is also fed by *all-trans* retinol derived from the photolysis of rhodopsin in the rod outer segments and from the blood supply. For de novo synthesis as well as for regeneration of rhodopsin the retinylesters are hydrolyzed and both *all-trans* and *11-cis* retinol move towards and through the outer segments. The *11-cis* configuration may be lost during this migration through nonspecific isomerization to the *all-trans* form (Daemen et al., 1974). Subsequently, the *all-trans* retinol is oxidized by the outer segment retinoldehydrogenase to *all-trans* retinaldehyde, which is reisome-

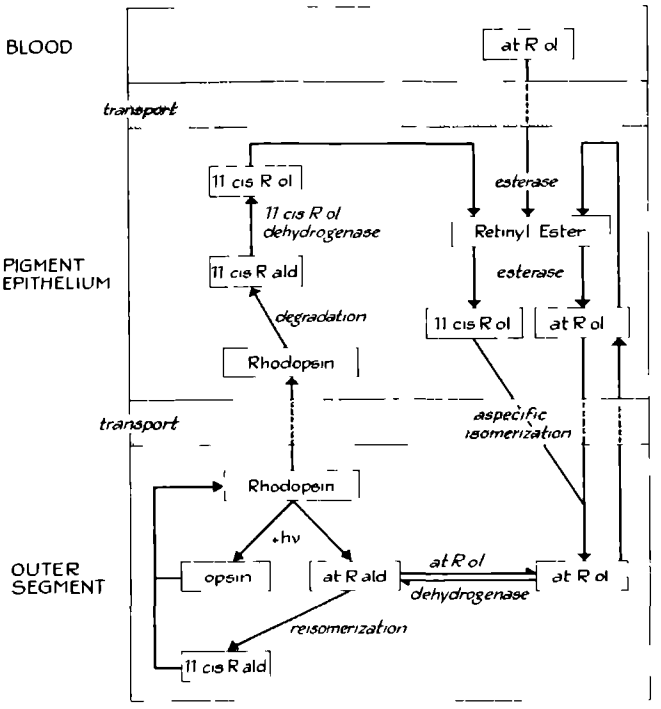

Fig. 29 Schematic representation of pathways of vitamin A compounds in the bovine eye.
Abbreviations: at R-ol: *all-trans* retinol;
at R-ald: *all-trans* retinaldehyde.

rized to the *11-cis* configuration and the resulting *11-cis* retinaldehyde recombines with opsin. The latter step drives the entire series of reactions by trapping the final product, *11-cis* retinaldehyde (Wald and Hubbard, 1950; Wald, 1968).

According to this formulation, summarized in fig. 29, the function of the retinoldehydrogenases in the eye would be to permit recycling of retinaldehyde for visual pigment formation, thereby efficiently conserving the ocular stock of vitamin A compounds essential for visual function.

CHAPTER 7

DISTRIBUTION OF RETINOLDEHYDROGENASE ACTIVITIES IN SUBCELLULAR FRACTIONS OF BOVINE RETINA AND PIGMENT EPITHELIUM

7.1 Introduction

In chapter 6 we have discussed the possible role of the rod outer segment retinoldehydrogenase and the pigment epithelial retinoldehydrogenase isolated in cattle and in rats.

Since the rod outer segment retinoldehydrogenase does not convert *11-cis* vitamin A compounds, we believe that the enzyme regulates the amount of *all-trans* retinaldehyde present in the rods by catalyzing the conversion of *all-trans* retinol to *all-trans* retinaldehyde during dark adaptation and the reverse reaction during light adaptation. This assumption is reasonable, if there is no other retinoldehydrogenase able to convert *11-cis* compounds present in the rod.

The retinoldehydrogenase present in the pigment epithelium presumably functions in the degradation of rhodopsin in this tissue by reducing its *11-cis* retinaldehyde to retinol. The degradation of rhodopsin is associated with lysosomal activity. A localisation of the retinoldehydrogenase near the lysosomes would support the hypothesis concerning its function. There may also be other retinoldehydrogenases present in the pigment epithelium. We have tried to test the last two points and to exclude the presence of *11-cis* compound converting enzymes in the retina by studying the subcellular distribution of retinoldehydrogenases. We have fractionated bovine retina and pigment epithelium by isopycnic centrifugation in sucrose density gradients, and have assayed the subcellular fractions for retinoldehydrogenase activity and for enzyme activities known to be associated with lysosomal and microsomal particles.

7.2 Materials and Methods

7.2.1 Tissue fractionation

Subcellular fractions of bovine retina and pigment epithelium are obtained by two procedures. In procedure I the retina of each cattle

eye is carefully removed in dim red light, and the pigment epithelium is scraped off the choroid. These tissues are then homogenized in 0.3M sucrose buffered with 30 mM Tris-acetate (pH 7.3) and fractionated as described in fig. 30. The tissue homogenate is subjected to a low speed centrifugation (5 min., 500 x g) to remove nuclei and unbroken cells. The resulting supernatant is subjected to a density gradient centrifugation, in which 20 fractions are collected for an accurate comparison of the distribution of the retinoldehydrogenases with that of rhodopsin.

In procedure II the discs are trepnined from opened eyes with a 1 cm diameter trephine in the light. The retinal and pigment epithelial layers (including choroid) are peeled off from the discs and are homogenized in 0.3 M sucrose-30 mM Tris-acetate buffer (pH 7.3) and fractionated according to fig. 31. This procedure omits the low speed centrifugation of the tissue homogenate in order to permit estimation of total enzyme activities. In this case only 5 broad fractions are removed, which are sufficiently large to compare the total and specific activities of the retinoldehydrogenases with those of the marker enzymes for the subcellular particles. The five fractions obtained by this procedure are analyzed for glucose-6-phosphatase and for both β-galactosidase and β-glucosidase activities. Glucose-6-phosphatase is generally accepted as a marker enzyme for the microsomal fragments representing the endoplasmic reticulum (Nordlie and Arion, 1966; Hinton, 1969), while β-galactosidase and β-glucosidase are acknowledged as marker enzymes for lysosomes (Beck and Tappel, 1968).

7.2.2 Assay of enzyme activities

Retinoldehydrogenase: The tissue fraction is suspended in 0.5 ml 66 mM Na-acetate (pH 5.0), to which 10 µl of a 50 mM co-enzyme solution in the same buffer (NADH when *11-cis* retinaldehyde is the substrate; NADPH when *all-trans* retinaldehyde is the substrate) and 10 µl of a 6 mM retinaldehyde solution in ethanol are added. The amount of retinaldehyde remaining after incubation is determined by the thiobarbituric acid method of Futterman and Saslaw (1961).

Glucose-6-phosphatase: The method of Nordlie and Arion (1966) is used. The tissue fraction is suspended in 0.075 ml 0.1 M Na cacodylate buffer (pH 6.5), to which 0.01 ml 10% (w/w) glucose-6-phosphate is added.

After 20 min. at 37° the reaction is stopped by adding 0.075 ml 15% (w/w) trichloroacetic acid, and the mixture is centrifuged for 20 min. at 17,000 g. Inorganic phosphate is determined by the method of Bonting (1970).

β-Glucosidase: The tissue fraction is suspended in 0.9 ml of a 0.1 M citrate-0.2 M NaH_2PO_4 buffer (pH 5.0), to which are added 0.02 ml 5% Triton X-100 and 0.1 ml 0.1 M 4 methyl umbelliferyl-D-glycopyranoside.The mixture is incubated for 1 hr at 37°C, and the reaction is stopped by addition of 2.0 ml 0.4 M glycine-NaOH buffer (pH 10.3). The amount of umbelliferone released by the enzyme is determined by its 454 nm fluorescence excited by 360 nm light (Robinson et al., 1967; Robins et al., 1968).

β-Galactosidase: The same procedure as for β-glucosidase is used, except that the citrate-phosphate buffer has a pH 4.5 and the substrate is 4-methyl umbelliferyl-D-galactopyranoside (Robinson et al., 1967; Robins et al., 1968).

Rhodopsin: The fraction is suspended in 1% Triton X-100, containing 50 mM hydroxylamine (pH 6.5). The rhodopsin content is determined by measuring the 500 nm absorbance before and after illumination of the sample.

Protein: the method of Lowry et al. (1951) is used with bovine serum albumin serving as the standard.

All data reported here are based on assays in which the rate of substrate conversion is constant with time and is proportional to the amount of enzyme added.

7.3 Results

Fig. 32 represents the results of rhodopsin measurements and retinoldehydrogenase assays with *all-trans* retinaldehyde and *11-cis* retinaldehyde as substrates in 20 retina fractions, collected according to procedure I (fig. 30). The enzyme activity towards *all-trans* retinaldehyde coincides closely with the distribution of rhodopsin in the gradient. Below the rod outer segment layer another band of retinoldehydrogenase activity is present, which does not coincide with rhodopsin. This band probably represents large microsomes from rod inner segments, as is indi-

PROCEDURE I

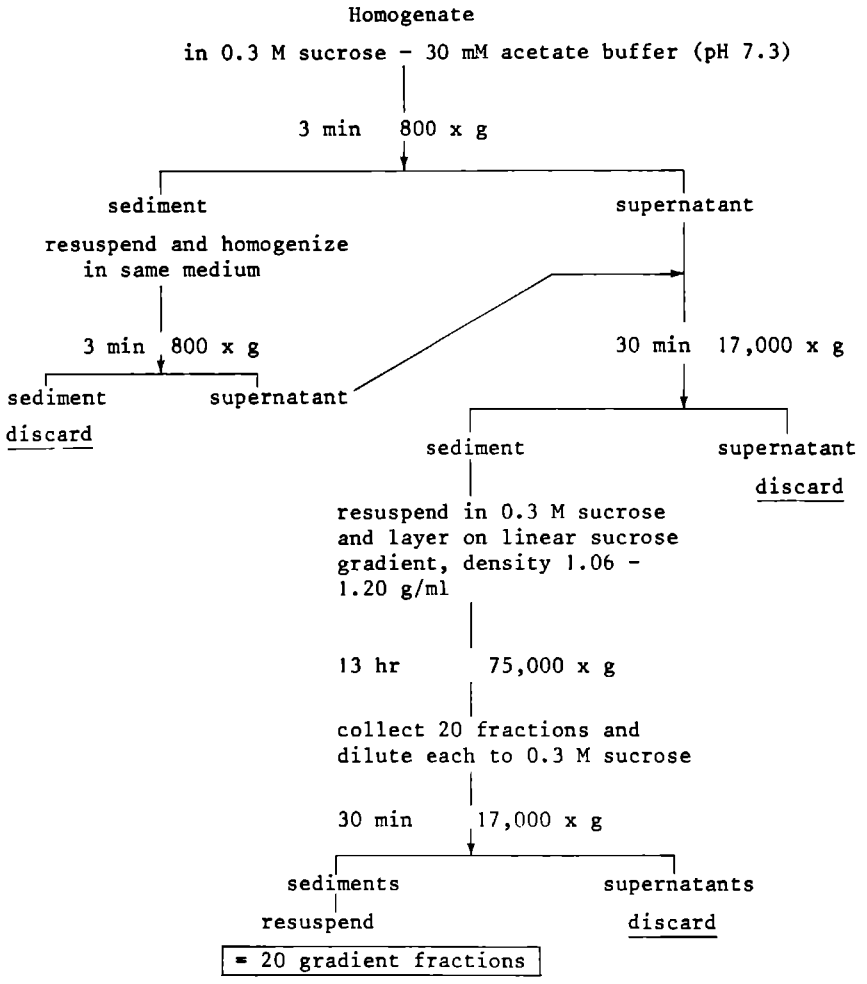

Fig. 30 Procedure I for subcellular fractionation of bovine retina and pigment epithelium by isopycnic centrifugation in linear sucrose gradients.

PROCEDURE II

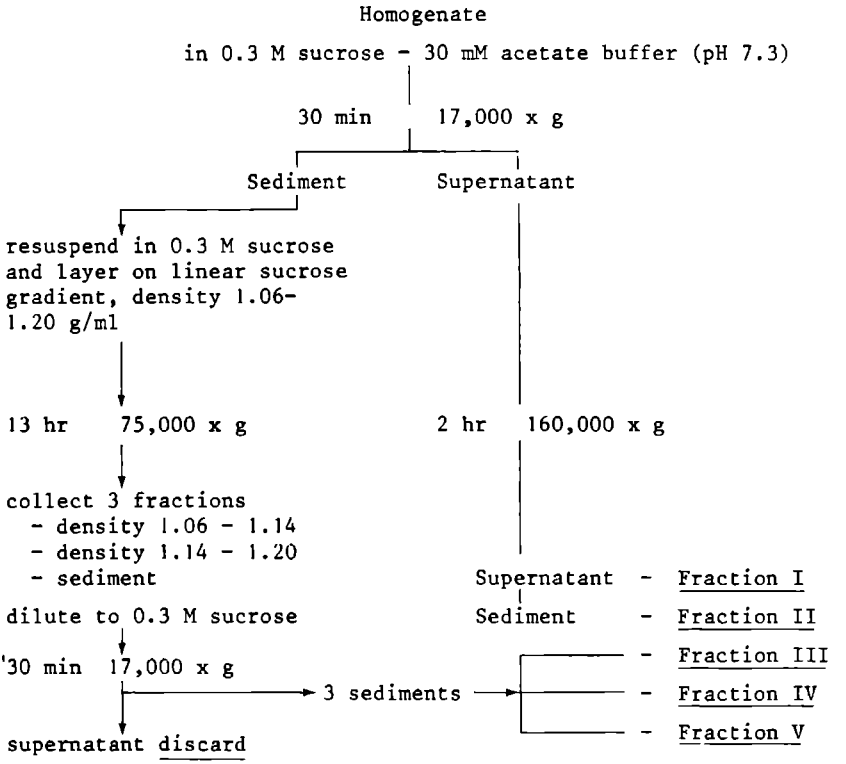

Fig. 31 Procedure II for subcellular fractionation of bovine retina and pigment epithelium by isopycnic centrifugation in linear sucrose density gradients

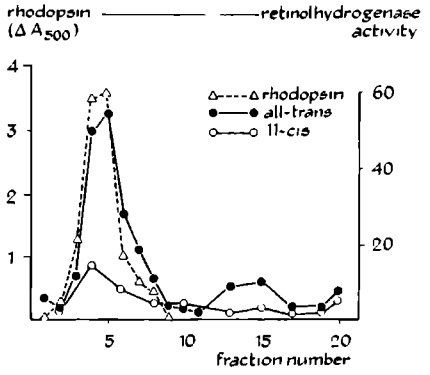

Fig. 32 Distribution of rhodopsin and of stereospecific retinoldehydrogenase activities in subcellular fractions obtained by fractionation procedure I from the retina. The sucrose gradient density varies linearly from 1.06 (fraction 2) to 1.20 (fraction 20). Retinoldehydrogenase activity is given in nmol retinaldehyde reduced per minute.

cated by the high activities of ß-glucosidase, ß-galactosidase and glucose-6-phosphatase activities (see fig. 33). We have tested the enzymatic activity of this band towards *all-trans* and *11-cis* compounds by thin layer chromatography. It has the same characteristics as rod outer segment retinoldehydrogenase and is not able to convert *11-cis* vitamin A compounds. The slight *11-cis* retinaldehyde reduction observed in the assay is probably the result of aspecific isomerization of the substrate.

Fig. 34 represents the distribution of rhodopsin and *all-trans* and *11-cis* retinaldehyde reduction capacity in the pigment epithelial fractions. The rhodopsin in the fractions is probably partly due to contamination of the pigment epithelium with rod outer segments outside the pigment epithelial cells, and partly to phagocytized rod outer segment membranes (Shichi, 1973). The maximal *all-trans* retinaldehyde reduction coincides with the rhodopsin peak. The *11-cis* retinaldehyde reduction is more or less evenly distributed in the fractions. The denser rhodopsin containing particles (fraction 5 and 6) show an increase in *11-cis* retinoldehydrogenase activity and a decrease in *all-trans* retinoldehydrogenase activity.

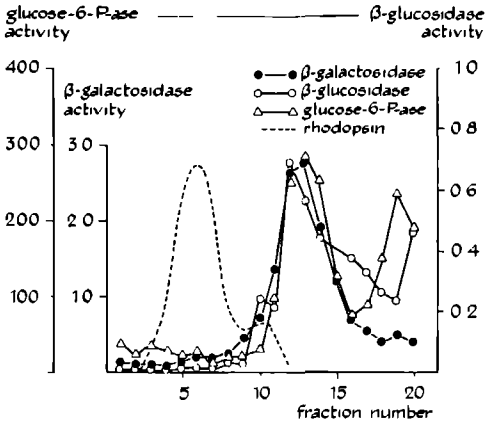

Fig. 33 Distribution of β-glucosidase and β-galactosidase activities in subcellular fractions obtained by fractionation procedure I from the retina. The sucrose gradient density varies linearly from 1.06 (fraction 2) to 1.20 (fraction 20). The glucosidase and galactosidase activities are given in mmol substrate hydrolyzed per hour, the glucose-6-phosphatase activity given in nmol substrate hydrolyzed per minute. For comparison with fig. 32 the distribution of rhodopsin is also shown.

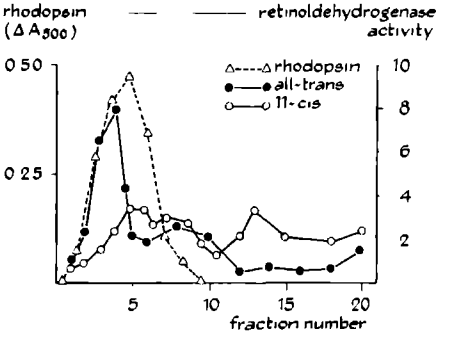

Fig. 34 Distribution of rhodopsin and stereospecific retinoldehydrogenase activities in subcellular fractions obtained from pigment epithelium by fractionation procedure II. The sucrose gradient density varies linearly from 1.06 (fraction 2) to 1.20 (fraction 20). Retinoldehydrogenase activity is given in nmol retinaldehyde reduced per minute.

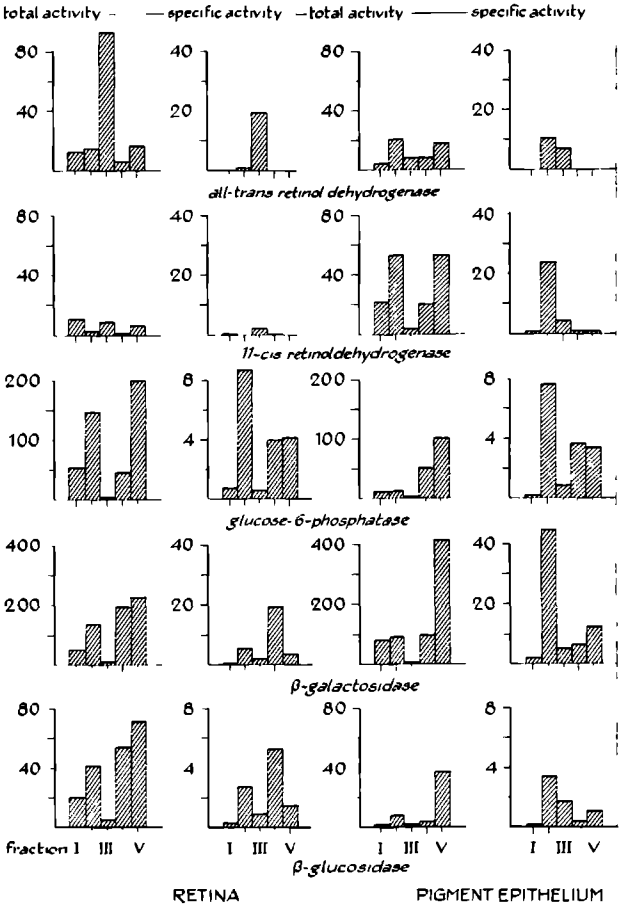

Fig. 35 Total and specific activities of stereospecific retinol-dehydrogenases and of microsome and lysosome associated enzymes in subcellular fractions obtained from equal areas of retina and underlying pigment epithelium by fractionation procedure II (fig. 31).

Fraction I = supernatant
Fraction II = "microsomes"
Fraction III = rod outer segments
Fraction IV = below rod outer segments
Fraction V = sediment

Retinaldehyde reduction activities are expressed in nmol retinaldehyde reduced per minute. The marker enzyme activities are expressed in nmol substrate hydrolyzed per minute. Specific activities are expressed in total activity per mg protein.

Fig. 35 represents the total and specific activities of *all-trans* retinaldehyde reduction, *11-cis* retinaldehyde reduction and assays on the marker enzymes glucose-6-phosphatase, β-galactosidase and β-glucosidase in whole retina and pigment epithelium homogenates, separated in five fractions by procedure II (fig. 31). The subcellular distribution of the enzymes permit several conclusions. The highest total and specific *all-trans* retinaldehyde reduction in retina as well as in pigment epithelium are found in fraction III, which contains rod outer segment material. The total and specific activities of the microsomal and lysosomal marker enzymes are very low in this fraction. *11-cis* retinaldehyde reduction is found almost entirely in the pigment epithelium with the highest specific activity present in fraction II. In this fraction the specific activities of the marker enzymes are high, suggesting that fraction II is mainly derived from endoplasmic reticulum and lysosomes. In fraction III, which contains the rod outer segments, the *11-cis* retinaldehyde reduction is virtually absent. The specific activities of the lysosomal marker enzymes β-galactosidase and β-glucosidase in fraction III of the pigment epithelium are slightly higher than the specific activities of these enzymes in the corresponding retinal fraction, suggesting that this fraction contains phagocyted rod outer segment material in the process of digestion by lytic enzymes from phagosomes.

7.4 Discussion

Although the data shown in fig. 32 - 35 are from experiments in which the rate of substrate conversion was constant with time and varied linearly with enzyme concentration, there are reasons for refraining from too detailed comparison of the activities. First, the enzyme preparations are impure, and enzyme activities are consequently subject to inhibitors and activators which may be introduced by homogenization. Secondly, the homogenization procedure may affect the retina and the pigment epithelium differently, so that fractions of equal density from the two tissue homogenates may contain different mixtures of subcellular particles.

Nevertheless, a few general conclusions concerning the subcellular distribution of ocular retinoldehydrogenases can be made. The total and

specific activities towards *all-trans* retinaldehyde are highest in the outer segment fractions derived from the retina and the pigment epithelium. The total and specific activities of the microsomal and lysosomal enzymes are among the lowest in these fractions. These results confirm that this enzyme activity occurs predominantly in the rod outer segments.

The total activity towards *11-cis* retinaldehyde in the pigment epithelium is five times that in the retina, and the highest specific activity of the enzyme is found in the microsomal fraction of the pigment epithelium. It is also clear that the enzyme is virtually absent from the fraction containing the rod outer segments. These results show that the *11-cis* preferring retinoldehydrogenase is predominantly an activity of the pigment epithelium and that it is associated with a membrane fraction distinct from the rod outer segments. This fraction has also the highest specific activity of the microsomal and lysosomal marker enzymes, suggesting that this enzyme may be connected with digestive activity in the pigment epithelial cell. But it cannot be determined with certainty whether this fraction represents primarily lysosomes, endoplasmic reticulum or another subcellular structure. This can only be determined by more complete separation of the subcellular fractions.

The increase in *11-cis* specific activity and decrease in *all-trans* specific activity in the fractions just below that with the highest rhodopsin content in the pigment epithelium (fig. 34) may be tentatively explained by assuming that these fractions contain more dense particles containing rhodopsin in various stages of digestion i.e. phagosomes. During digestion the rod outer segment membranes loose their *all-trans* retinoldehydrogenase-activity while the *11-cis* retinoldehydrogenase-activity increases due to fusion of lysosomes with the phagosomes.

Fig. 35 shows that the *all-trans* and *11-cis* specific retinol dehydrogenase activities are not limited to the rod outer segment fractions and the pigment epithelial microsome fractions respectively. This is not quite unexpected for the gradient sediment fraction. This fraction is obtained without a low speed centrifugation to remove nuclei and unbroken cells. The presence of nuclei and unbroken cells in the gradient sediment may explain the high total activity and low specific activity of the retinoldehydrogenases as well as of the other enzymes.

As for the *11-cis* retinoldehydrogenase activity in the rod outer

segment, it is known that the *11-cis* isomers of vitamin A compounds are unstable and are isomerized non-specifically to the *all-trans* form (Daemen et al., 1974; Futterman and Futterman, 1974). Thus, some of the *11-cis* retinaldehyde used as substrate in these experiments was probably isomerized to the *all-trans* configuration, and then reduced by the *all-trans* specific enzyme.

The presence of high *all-trans* as well as *11-cis* retinaldehyde specific activity in the "microsome" fraction of the pigment epithelium is probably due to the incomplete stereospecificity of the *11-cis* retinoldehydrogenase: the ratio of initial velocities of the reactions with *11-cis* versus *all-trans* retinaldehyde is 1.4 (see chapter 6, section 5.4).

Finally, the retinoldehydrogenase activity in the supernatants may be due to partial solubility of the enzyme under our homogenization and fractionation conditions or to incomplete sedimentation of membrane particles.

We can conclude that the retina contains no significant amounts of retinoldehydrogenase activity towards *11-cis* vitamin A compounds. Most of the retinoldehydrogenase activity is bound to the rod outer segments. A small amount of *all-trans* retinoldehydrogenase activity seems not to be associated with rhodopsin but with a fraction presumably containing rod photoreceptor inner segments. This activity may very well represent the same enzyme as the rod outer segment bound enzyme, synthetized in the rod inner segment prior to the assembly of the membrane components in the rod outer segment membrane.

The pigment epithelium contains also *all-trans* retinaldehyde reduction activity associated with rod outer segment material, but the highest activity towards retinaldehyde with a strong preference for *11-cis* retinaldehyde is found in a microsomal fraction, which, according to the marker enzyme activities, may very well contain the lysosomes of the pigment epithelium. This reinforces the hypothesis concerning the digestive role of this retinoldehydrogenase.

CHAPTER 8

BIOCHEMICAL ASPECTS OF INHERITED RETINAL DYSTROPHY IN THE RCS RAT *

8.1 Introduction

Rats with the inherited retinal dystrophy first described by Bourne et al. (1938) show a progressive structural and functional degeneration of the photoreceptor layer in the retina from the twelfth day onwards (see chapter 3, section 3.1).

The structure of the rod outer segment membranes in these animals and various enzymes related to the metabolism of rods and pigment epithelium have been studied for possible defects by many investigators. Their results are described in chapter 3, section 3.1. Although several changes have been found, the primary biochemical lesion is still not known. Even the location of the primary biochemical defect is still disputed. It may be located in the pigment epithelium, leading to a disturbance of rod phagocytosis or certain metabolic activities, or it may be located in the rod inner segments, leading to a disturbance in rod outer segment structure. This chapter is concerned with various possible metabolic defects in pigment epithelium and rod receptors.

In view of the important role of vitamin A metabolism and the ocular retinoldehydrogenases in photoreceptor metabolism, we have investigated various properties of rhodopsin in the dystrophic rat and we have correlated the results with the retinoldehydrogenase activity in the rod outer segment. We have studied the effect of continuous dark adaptation during the first 40 days of life on the development of the dystrophic rat retina, as compared to maintenance in normal light conditions. We have also investigated another aspect of rod outer segment metabolism important in visual excitation, the Na-K-activated ATPase activity. This activity has been reported to be progressively deficient in retinas of dystrophic rats (Bonavita et al., 1966, 1967). According to current insight this enzyme system is thought to be located in the inner segment of the photo-

* I am very grateful to mr. P.A.A. Jansen for his skilful execution of the rhodopsin measurements and retinoldehydrogenase assays and to Dr. R.M. Broekhuyse for his assistance in the fatty acid analysis described in this chapter.

receptor cell and to be responsible for generating the dark current needed for its excitability by light. Finally we have analyzed the fatty acid composition of normal and dystrophic rat retinas. In view of the large amount of highly unsaturated lipids in the rod outer segment membranes (Daemen, 1973), we have investigated whether there exist any differences in the fatty acid composition of normal and dystrophic retinas.

8.1.1 Regeneration capacity of rhodopsin

Dowling and Sidman (1962) have found a striking influence of illumination on the rhodopsin content of dystrophic rat retinas. Dystrophic animals raised in the dark had at 30 days of age twice as much rhodopsin as the normal rats. When raised in the light, the amount of rhodopsin present reached a lower maximum at about 26 days of age and then dropped to values far below the normal control values. Hence the light causes a fast disappearance of rhodopsin in the dystrophic rat retina. This suggests at least a partial inability to resynthesize rhodopsin after bleaching, due either to a disruption of the normal metabolic pathways of vitamin A compounds, or to an abnormality in the rhodopsin molecule itself. We have therefore investigated whether a defect in the regeneration capacity of the visual pigment in the dystrophic retina could be involved in the degeneration process. The ability of the visual pigment to regenerate in vitro after bleaching and incubation with exogenous *11-cis* retinaldehyde has been tested.

8.1.2. Retinoldehydrogenase activities

Since retinoldehydrogenases in the retina, and probably in the pigment epithelium as well, are of crucial importance in the vitamin A metabolism of the photoreceptor system, we have investigated the relationship of rhodopsin content and retinoldehydrogenase activities in the retina and in the pigment epithelium during retinal development in normal and dystrophic animals. Animals kept in darkness as well as animals reared under cyclic light and dark conditions have been studied. Notwithstanding the previous studies of the rhodopsin content by Dowling and Sidman (1962) and those of the retinoldehydrogenase activity by Reading and Sorsby (1966), a carefully controlled study of these two

parameters in normal and dystrophic rats under different illumination conditions appears necessary. Only such a study will allow to determine whether any deviations in these two parameters exist in the rod outer segment membranes of the dystrophic rat. The pigment epithelium retinol-dehydrogenase activity in the dystrophic rat has also been studied because a defect in its function might very well explain the inability of the pigment epithelium of the dystrophic rats to degrade photoreceptor outer segment membranes. Rhodopsin, in contrast to opsin, is actually a very stable molecule which in vitro can only be degraded by proteolytic enzymes after previous solubilization in detergents (van Breugel et al., 1974). It might be that removal and reduction of the chromophoric group *11-cis* retinaldehyde is necessary before degradation of the lipoprotein can take place. A deficiency of retinoldehydrogenase in the pigment epithelium could, therefore, block digestion of the visual pigment and thus of the photoreceptor membranes.

8.1.3 Na-K-activated adenosine triphosphatase activity

Investigation of some enzyme activities in normal and dystrophic rat retinas led Bonavita et al. (1966, 1967) to suggest that a deficiency in Na-K-activated ATPase might be the primary biochemical lesion in this hereditary disease. While they did not find any difference in retinal Na-K-ATPase activity in 2-day old rats, they reported a progressive decrease in dystrophic retinas after the 10th day, leaving only 13% of the normal Na-K-ATPase activity in 120-day old dystrophic rats. They reported no differences in the Na-K-insensitive Mg-ATPase activity between normal and affected animals.

Reinvestigation of this matter seemed worthwhile for two reasons. First, the specific activities reported by Bonavita et al. (1966, 1967) are uncommonly high. Their values would come to 100 - 300 moles ATP converted per kg dry weight per hr, assuming a protein content of 60%. Values given in the literature for Na-K-ATPase activities of retinas of various species (human and cat, Bonting et al., 1961; cow, rabbit and frog, Bonting et al., 1963a; pig, Frank and Goldsmith, 1965; rat, Langham et al., 1967) vary between 2 and 11 moles per kg dry weight per hr. The highest values reported for any tissue are 12.1 for the distal tubules of the rat nephron (Schmidt and Dubach, 1969), to 13.4 for the salt gland

of the herring gull (Bonting et al., 1964) and to 18.4 for the non-innervated membranes of the electric organ of Electrophorus electricus (Bonting, 1970, pp. 330-333).

Secondly, it is unlikely that the Na-K-ATPase activity of rats with inherited retinal dystrophy would be so dramatically decreased. Though the photoreceptor layer of these animals has almost completely disappeared at the age of 60 days, the bipolar and ganglion cell layers are essentially well preserved (Dowling and Sidman, 1962). One would therefore expect a smaller drop in Na-K-ATPase activity, since the photoreceptor layer on a weight basis constitutes only 15% of the whole retina.

8.1.4 Fatty acid composition

It is known that vertebrate rod outer segment membranes contain a high proportion of long-chain highly unsaturated fatty acids, which gives the membranes a fluid character (Daemen, 1973). If some of these fatty acids are deficient in dystrophic animals, the membrane structure of the rod outer segments might be altered. Such a deficiency could occur through an inability to synthesize them, or through a deficiency in the mechanisms protecting them from oxidation. It might result in an inability of the pigment epithelium to phagocytize the rod outer segments. The pigment epithelial phagocytic activity is probably very sensitive to alterations of the rod plasma membrane or disc membrane structure (Custer and Bok, 1975). Being unable to separate rod outer segments from rat retinas in sufficient amounts, we have used whole retina homogenates from normal and dystrophic rat retinas and analyzed these homogenates for fatty acid composition.

8.2 Materials and Methods

8.2.1 Materials

The rats in these experiments are descendants of the strain described by Bourne, Campbell and Tansley (1938). Albino rats of the Wistar strain are used as controls. Histological sections of the eyes of normal and dystrophic animals clearly indicate the extensive degeneration of the photoreceptor layer in the latter animals. Experimental animals are killed by cervical dislocation, the eyes are enucleated and after removal

of cornea, lens and vitreous, the retinas are collected. The eye cups, containing pigment epithelium, choroid and sclera are collected separately.

All-trans retinaldehyde is obtained from Eastman Rochester N.Y.. The *11-cis* retinaldehyde, used in regeneration experiments and retinoldehydrogenase assays is prepared by photo-isomerization of an *all-trans* retinaldehyde solution in ethanol and elution of the *11-cis* isomer on an aluminum oxide column with benzene/hexane (1:9 V/V).

NADH and NADPH are obtained from Boehringer, Mannheim, Germany.

8.2.2 Methods

In the experiments on rhodopsin measurements and on retinoldehydrogenase activities dystrophic rats and normal control rats are kept either in darkness or in cyclic light (12 hours darkness, 12 hours illumination by two fluorescent tubes of 40 W at a distance of about 2 m) from three days after birth.

Determination of rhodopsin and its regeneration capacity: Four retinas are homogenized in 0.6 ml 0.16 M Tris/HCl pH 7.2. The rhodopsin content is determined by mixing 200 µl homogenate with 50 µl 10% (w/v) Triton X-100 and 10 µl 1 M hydroxylamine and recording the 500 nm absorbance before and after illumination. The remaining homogenate is illuminated for 30 minutes with a 300 W tungsten source behind an orange filter, which transmits only wavelengths longer than 550 nm, and an infrared filter to avoid thermal decomposition. In the dark 20 nmol *11-cis* retinaldehyde in 10 µl methanol is added and the mixture is incubated for 30 minutes at room temperature. After incubation the rhodopsin content of the sample is determined as described before.

Enzyme preparation: For the preparation of rod outer segment retinoldehydrogenase 16 retinas are collected in ice-cold 0.16 M Tris/HCl pH 7.2 and homogenized. The suspension is centrifuged for 20 minutes at 20,000 x g and 4°C and the sediment is resuspended in 1 ml 0.1 M acetate buffer. For the preparation of pigment epithelium retinoldehydrogenase 16 eye cups containing the pigment epithelium are collected in ice-cold 0.25 M sucrose - 1 mM EDTA - 0.01 M Tris/HCl (pH 7.8) and homogenized with a Polytron homogenizer for 10 seconds. The homogenate is centrifuged for 5 minutes at 10,000 x g and 4°C. The sediment is again homoge-

nized in the same way and centrifuged for 1 hour at 100,000 x g and 4°C. The resulting sediment is washed two times with 0.1 M acetate buffer (pH 5.0) and ultimately suspended in 1 ml 0.1 M acetate buffer.

Retinoldehydrogenase assay: Retinoldehydrogenase activity is determined by measuring the velocity of substrate conversion during the first 3 min. of incubation. After adding 50 nMol retinaldehyde in 10 µl methanol to 400 µl enzyme suspension, the mixture is incubated at 37°C. At time zero 250 nMol NADPH is added and the decrease of retinaldehyde is measured as a function of time by using the method of Futterman and Saslaw (1961). The rod outer segment retinoldehydrogenase activity is determined with *all-trans* retinaldehyde as substrate, the pigment epithelium retinoldehydrogenase with *11-cis* retinaldehyde as substrate.

Na-K-ATPase activities: Retinas of normal and dystrophic rats raised under ordinary laboratory illumination conditions are frozen, lyophilized and stored in vacuo at -20°C. Samples of the lyophilized material are weighed on a Cahn electrobalance and homogenized in a known volume of distilled water (0.2% dry wt./vol.). Aliquots of these homogenates are used for the ATPase assay (Bonting et al., 1963b). The properties of the enzyme are determined as described by Bakkeren and Bonting (1968). For most experiments only media A (complete) and E (no K^+ and 10^{-4}M ouabain added) are used. The activity in medium E gives the Mg-ATPase activity and the difference between media A and E gives the Na-K-ATPase activity.

Fatty acid analysis: Retinas of 40 days old normal and dystrophic rats, raised under ordinary laboratory illumination conditions are collected (1957). Fatty acid methylesters of total lipids are prepared with boron trifluoride according to Morrison and Smith (1964). Identification and quantification of the fatty acids are performed by gas chromatography on a 10% ethylene glycol succinate column (8 ft x 0.25 inch) and on a 3% SE-30 column (6 ft x 0.125 inch) at 200°C. The nitrogen flow rates are 50 ml/min. and 13 ml/min. respectively.

8.3 Results

8.3.1 Rhodopsin content and its regeneration capacity

In normal adult animals the amount of rhodopsin present in the

retina is about 1.3 nmol per retina, which regenerates for about 90%. In dystrophic animals reared in the dark the rhodopsin content rises steadily till it reaches a maximum of 2.8 nmol rhodopsin per retina at about 34 days of age (fig. 36). A constant regeneration percentage of about 70% is found. In dystrophic animals reared under cyclic light and dark conditions, the rhodopsin content per retina reaches a maximum of 2.5 nmol per retina at about 25 days of age, after which the rhodopsin content falls sharply to 0.4 nmol per retina at 40 days of age (fig. 37). In this case the regeneration percentage is not constant. In fact, after 28 days of age the regeneration percentage significantly exceeds 100%, suggesting that in these animals a substantial amount of opsin is present, possibly because insufficient *11-cis* retinaldehyde is available.

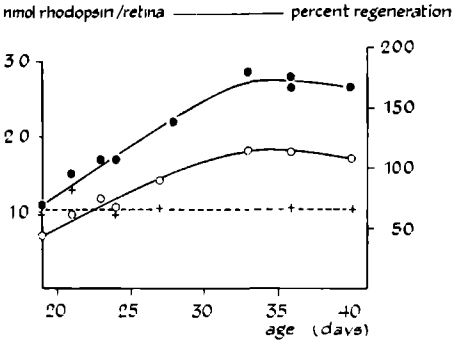

Fig. 36 Rhodopsin content of retinas of dystrophic rats, raised in darkness (●—●). Also shown is the amount of rhodopsin, which can be regenerated with exogenous *11-cis* retinaldehyde after extraction and bleaching (○——○). The dotted line (+----+) indicates the (expressed in percent) ratio regenerated rhodopsin versus the rhodopsin content.

8.3.2 Rod outer segment retinoldehydrogenase activity

The retinoldehydrogenase activity more or less parallels the curve for the rhodopsin content (fig. 38). It exceeds the normal level in normal rats (40 nmols retinaldehyde reduced per minute per eye) and reaches a maximum of about 53 nmol per minute per eye. In dystrophic rats reared in the light, from the age of 25 days onward, the retinol-

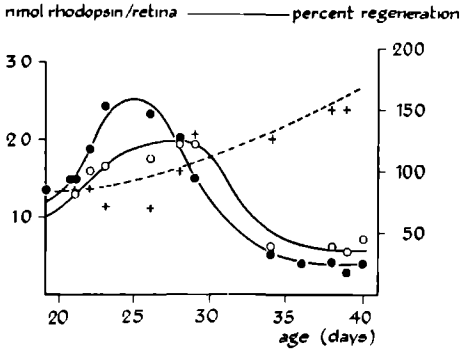

Fig. 37 Rhodopsin content of retinas of dystrophic rats, raised under normal conditions of illumination (●—●). Also shown is the amount of rhodopsin, which can be regenerated with exogenous *11-cis* retinaldehyde after extraction and bleaching (○—○). The dotted line (+----+) indicates the ratio regenerated rhodopsin versus the rhodopsin content (expressed in percent).

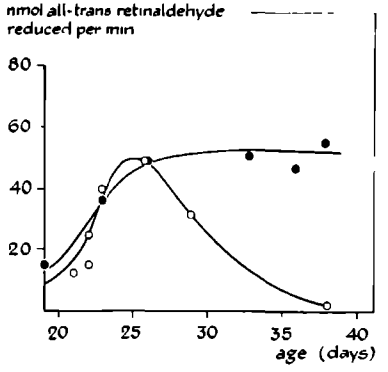

Fig. 38 Rod outer segment retinoldehydrogenase activity per retina in dystrophic rats raised in darkness (●—●) and in dystrophic rats raised under normal conditions of illumination (○—○).

dehydrogenase activity ceases and at 40 days almost no measurable enzyme activity is left. In dystrophic animals reared in the dark, the level of enzyme activity is still at its maximum.

8.3.3 Pigment epithelium retinoldehydrogenase activity

In the normal adult rat the activity of pigment epithelium retinoldehydrogenase is about 10 nmol per minute per eye. In the dystrophic rat the activities reach considerably higher levels (fig. 39). In animals kept in darkness, an activity of 35 nmol per minute per eye is reached in about 26 days, which thereafter remains constant. In animals kept under normal light conditions the curve of retinoldehydrogenase activity parallels the curve of the dark adapted animals, but after about 25 days the activity diminishes and reaches the normal level of 10 nmol per minute per eye at 35 days.

8.3.4 Na-K-ATPase activities

The properties of rat retinal Na-K-ATPase have been determined. It is maximally activated by Na^+ at a concentration of 50 mM, half-activated at 5 mM. Maximal activation by K^+ occurs at 5 mM, half-maximal activation at 1.1 mM. In the presence of 2 mM ATP Mg^{2+} is maximally active

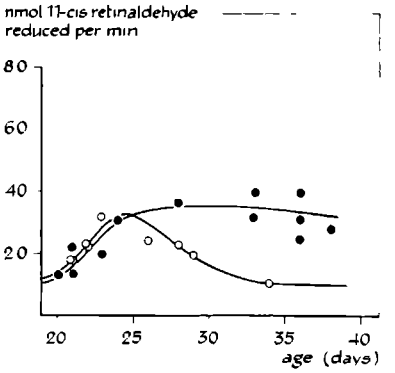

Fig. 39 Pigment epithelium retinoldehydrogenase activity per retina in dystrophic rats raised in darkness (●—●). and in dystrophic rats raised under normal conditions of illumination (○—○).

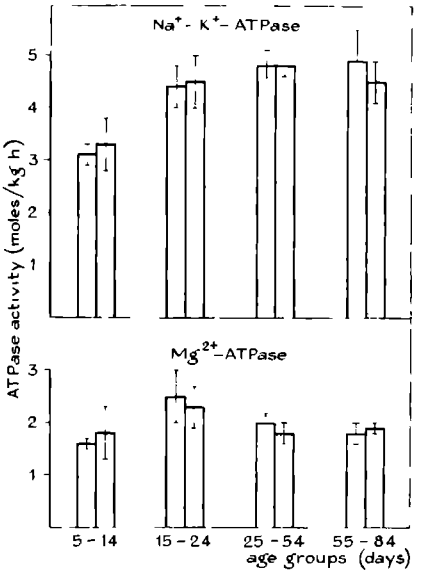

Fig. 40 Na-K-ATPase and Mg-ATPase activity in the developing retina of normal Wistar rats (open bars) and of rats with inherited retinal dystrophy (shaded bars). The length of the vertical line indicates twice the standard error.

at 2 mM and half-maximal activation occurs at 0.6 mM, while Mg-ATPase is maximally activated by Mg^{2+} at 4 mM and half-activated at 0.9 mM. The pH optima are 7.4 for Na-K-ATPase and 9.0 for Mg-ATPase. Na-K-ATPase shows a normal ouabain inhibition curve with a pI_{50} of 5.8 with complete inhibition at 10^{-3} M ouabain in the presence of 5 mM K^+. Inhibition with 10^{-4} M ouabain in the absence of K^+ (medium E) is also complete.

In fig. 40 the Na-K-ATPase and Mg-ATPase determinations in the retinas of four age groups of normal and dystrophic rats are summarized. Each bar represents a mean value obtained from at least nine retinas.

8.3.5 Fatty acid composition

The fatty acid composition of normal and dystrophic rat retinas is given in Table VI. The total quantities of fatty acids are equal in both assays. The only notable difference is for the 18:1 fatty acid, which comprises 9.8% in normal and 7.6% in dystrophic rats.

Table VI Fatty acid composition of normal and dystrophic rat retinas

Fatty acids	Distribution (g/100 g)	
	normal	dystrophic
16 : 0	17.4	17.9
16 : 1	8.1	8.4
18 : 0	21.8	21.7
18 : 1	9.8	7.6
18 : 2	0.8	0.7
18 : 3 20 : 0	0.4	0.4
20 : 1	0.2	0.2
21 : 0	0.3	0.3
20 : 4ω6	10.1	10.6
20 : 6ω3	31.1	32.2

8.4 Discussion

8.4.1 Rhodopsin and its regeneration

The rhodopsin content of the dystrophic rat retina during development in darkness or under normal light conditions has previously been investigated by Dowling and Sidman (1962). The difference in rhodopsin content between animals reared in darkness and animals reared in the light suggested to them that dystrophic animals are unable to resynthesize rhodopsin. We have therefore tested the regenerability of the rhodopsin after bleaching in vitro. We find higher rhodopsin contents than Dowling and Sidman (1962). This may be due to differences in the experimental methods. They treated the posterior half of the eye in 4% alum for 10 minutes, washed it twice in distilled water and once in phosphate buffer, and then extracted the rhodopsin with 2% digitonin overnight. We dissect the retina, homogenize it and extract rhodopsin with 1% Triton X-100. On the one hand, during dissection some retinal material (less than 10% in terms of rhodopsin) will be lost, on the other hand, dissection and homogenization make the rod outer segments more accessible

to the detergent solution. Triton X-100 is moreover a more effective detergent than digitonin. Apparently the latter two factors predominate, thus leading to a higher yield of rhodopsin in our experiments.

In normal rats about 90% of all rhodopsin present can be regenerated after bleaching in vitro, while in dystrophic rats reared in darkness only about 70% of the rhodopsin can be regenerated. In dystrophic animals reared in the light, at 25 days of age there is about 80% regeneration of rhodopsin but from 28 days onward the regeneration percentage significantly exceeds 100%, indicating appreciable amounts of opsin in the retina. The difference in rhodopsin regenerability in normal and dystrophic rat retinas suggests that the rhodopsin regeneration cycle in dystrophic animals is partially disturbed, possibly as a consequence of conformational changes in the rod outer segment membranes or in the rhodopsin molecule itself. This is probably not the only reason why the rhodopsin gradually disappears in the dystrophic rat. In the animals reared in the light, the retina from 28 days onward appears not to be able to regenerate all its opsin, although the opsin can regenerate in vitro. This effect may be due to the thick layer of membrane debris, which hinders the exchange of vitamin A compounds between photoreceptor and pigment epithelium.

8.4.2 Rod outer segment retinoldehydrogenase

Reading and Sorsby (1966) have investigated the retinoldehydrogenase activity during the development of normal and dystrophic rats. Their method is, however, not very suitable. They tested the oxidation of *all-trans* retinol by assaying the retinaldehyde content at zero time and after incubation for 30 and 90 minutes, in an incubation mixture containing 0.3% Tween 80, and with a very high pH (9.5). Both in cattle and in rats the oxidative activity is much lower than the reductive activity, which makes the measurement of oxidative activity in small amounts of tissue difficult. In addition, the pH of their medium was about one unit above the optimal value. There is also reason to believe that even small amounts of detergent seriously affect the enzyme activity (see chapter 5, section 3.1). Finally, they measured the enzyme activity after incubation for 90 minutes, without testing linearity with time. In our experience, it seems unlikely that linearity existed under their conditions.

We have measured the reduction activity of the enzyme towards *all-trans* retinaldehyde as described in 8.2.2, observing the enzyme activities during growth in darkness as well as under normal light conditions. The results parallel the findings in rhodopsin content. This is perhaps not surprising, because the enzyme is a constituent of the rod outer segment membranes which contain the rhodopsin.

8.4.3 Pigment epithelium retinoldehydrogenase

The retinoldehydrogenase present in pigment epithelium has not previously been investigated in the dystrophic rat. The very high activities towards *11-cis* retinaldehyde found in the posterior eye poles after removal of the retina follow more or less the same pattern as the rod outer segment retinoldehydrogenase. It is improbable that this activity is the result of reduction of *all-trans* retinaldehyde, aspecifically isomerized from *11-cis* retinaldehyde, by remnants of rod outer segment material. Firstly, most of the rod outer segment material is removed on dissection, and secondly in preparing the pigment epithelium homogenate all large cell fragments are sedimented by centrifugation, while the supernatant contains most of the enzymatic activity. The high enzymatic activities probably represent enzyme newly produced as a reaction to the large amounts of rod outer segment debris. A deficiency in this enzyme does clearly not occur in these dystrophic animals.

8.4.4 Na-K-activated adenosinetriphosphatase

From the data given in 8.3.4 it is clear that no significant differences in Na-K-ATPase or Mg-ATPase activity exist between normal and dystrophic retinas in the same age group. Furthermore, the activity values are all in the range mentioned by most authors so far (Bonting et al., 1961; Bonting et al., 1963a; Frank and Goldsmith, 1965; Langham et al., 1967). These experimental results indicate that there is no general Na-K-ATPase deficiency in the retina of rats with inherited retinal dystrophy. A localized deficiency in the outer segments of the receptor cells in the early stages cannot be completely ruled out, since these structures constitute only a very small part of the whole retina in this age range. This matter would require the application of microdissection and ultramicro assay techniques (Matschinsky, 1968) or histochemical staining

methods. Unfortunately, the former approach is extremely difficult in the early stage of development, and a reliable histochemical staining method for Na-K-activated ATPase does not yet exist (Bonting, 1970, pp. 305-306).

8.4.5 Fatty acid composition

From the data of table VI we can conclude that the fatty acid composition of retinas of normal rats is virtually equal to retinas of dystrophic animals. On an anatomical basis (see fig. 2) we judge that at least 10% of the fatty acids are derived from the photoreceptor cell outer segments. Therefore we may conclude that there are no gross differences in fatty acid composition between retinas of normal and dystrophic rats at an age of 40 days. At this age there must be defects in synthesis and probably also in protecting mechanisms against oxidation. Small but significant differences in fatty acid composition cannot be excluded by this method.

CHAPTER 9

GENERAL SUMMARY AND CONCLUSIONS

9.1 Introduction

In the preceeding experimental chapters we have tried to analyze some of the processes involving the function and metabolism of rhodopsin, in particular its chromophoric group vitamin A. These processes are rather exclusive to the retina. Hence, they may be important in understanding the metabolic errors of metabolism underlying certain hereditary retinal degenerations and drug-induced retinal diseases.

Three preliminary chapters deal with the normal anatomy (chapter 1) and physiology (chapter 2) of vertebrate photoreceptors and with data concerning the pathology of hereditary tapeto-retinal degenerations in man and experimental animals (chapter 3). In the subsequent chapters our experimental studies are described. We have studied some aspects of rhodopsin regeneration (chapter 4), of enzymes involved in vitamin A metabolism in the retina (chapter 5) and in the pigment epithelium (chapter 6), and of the intracellular localisation of these enzymes (chapter 7). Finally we have investigated processes concerning rod outer segment membrane and pigment epithelial function in animals with a hereditary retinal degeneration. In this context rhodopsin and its chromophore, vitamin A, the transport enzyme Na-K-Mg-activated ATPase and the fatty acid composition of the photoreceptor membranes have been studied.

9.2 Rhodopsin regeneration in the rat retina

Although regeneration of rhodopsin in the isolated rat retina has been claimed under certain conditions by Cone and Brown (1969), we have not been able to find any rhodopsin regeneration in the isolated rat retina, although we have carefully observed the conditions employed by Cone and Brown (1969). Addition to this system of factors, which might influence rhodopsin regeneration, does not result in regeneration. With this experimental model we are unable to solve the discrepancy between certain publications suggesting that the presence of the pigment epithelium is required for rhodopsin regeneration and other publications clai-

ming that rhodopsin regeneration can occur in the absence of the pigment epithelium.

From the results of chapter 5 about the stereospecificity of retinoldehydrogenase in the rod outer segment we conclude that isomerization of *all-trans* retinaldehyde to *11-cis* retinaldehyde must be able to occur in the rod outer segment itself. The role of the pigment epithelium in the isomerization reaction may consist in supplying an essential factor.

9.3 Retinoldehydrogenases in cattle and rat retina

The retinoldehydrogenase present in rod outer segment membranes has long been considered as an integrated component in the rhodopsin regeneration cycle (Wald, 1968). We have found that under no circumstances *11-cis* vitamin A compounds are products of conversion catalyzed by this enzyme. The small degree of conversion of *11-cis* vitamin A compounds in the presence of this retinoldehydrogenase probably takes place only after previous non-enzymatic isomerization to *all-trans* vitamin A compounds. This implies that *11-cis* retinaldehyde required for rhodopsin regeneration is formed directly from *all-trans* retinaldehyde in the rod outer segment. A conversion of *11-cis* retinol to *11-cis* retinaldehyde in the rod outer segment is excluded by the stereospecificity of the retinoldehydrogenase, and we have not found another retinoldehydrogenase in the rod outer segment. It is improbable that *11-cis* retinaldehyde moves from another localisation, e.g. the pigment epithelium to the rod outer segment, because the very reactive aldehyde group will prevent migration of the molecule. On the basis of these results we propose a simplified rhodopsin regeneration cycle. This still does not answer the question in which way isomerization of vitamin A aldehyde is achieved and whether the pigment epithelium is involved in the process.

Investigation of the enzymatic activity towards vitamin A compounds in pigment epithelium has shown the presence of a retinoldehydrogenase not previously described. This enzyme has a preference for the conversion of *11-cis* vitamin A compounds, and is definitely different from that in the rod outer segment retinoldehydrogenase. This difference consists in its different stereospecificity towards vitamin A compounds, but also in differences in stability, co-enzyme dependency and intra-

cellular localisation. It seems unlikely that the pigment epithelium enzyme is related directly to rhodopsin regeneration. It is, however, likely that this enzyme plays a role in the digestion of rod outer segment membranes by reducing the visual pigment chromophore *11-cis* retinaldehyde to retinol. This can then be recycled in ocular vitamin A metabolism and be utilized for resynthesis of rhodopsin.

The results of the experiments described in chapter 5 and 6 concerning the specificity of ocular retinoldehydrogenases have also been published elsewhere (F. Lion, J.P. Rotmans, F.J.M. Daemen and S.L. Bonting, 1975).

The question arises whether the two retinoldehydrogenases are the only ones important in retinal vitamin A metabolism. Additional support is desirable for the hypothesis that the pigment epithelial retinoldehydrogenase is involved in the degradation of rod outer segment membranes. We have, therefore, used tissue fractionation methods and analysis of the nature of the fractions by means of marker enzyme assays.

In the retina two localisations of retinoldehydrogenase activity have been established, one related to the rod outer segment membranes and another in a fraction which probably contains rod inner segment material. Both retinoldehydrogenase activities are restricted to *all-trans* vitamin A compounds. Presumably the retinoldehydrogenase activity in rod inner segment material represents the same enzyme as the rod outer segment bound enzyme, synthesized in the rod inner segment prior to assembly in the rod outer segment membrane.

The retinoldehydrogenase activity in the pigment epithelium appears to be largely present in a microsomal fraction, which is also rich in hydrolases. This indicates a possible relationship with lysosomal activity, and this supports the hypothesis concerning its role in rod outer segment membrane digestion.

The results of the experiments described in chapter 7 concerning the intracellular distribution of ocular retinoldehydrogenases have also been published elsewhere (W.F. Zimmerman, F. Lion, F.J.M. Daemen and S.L. Bonting, 1975).

9.4 Biochemical aspects of tapetoretinal degeneration in the rat

Although investigations of pathological conditions in animals are

of limited value only to the understanding of disease in man, the close similarity of retinal structure and function in all vertebrates gives the study of a hereditary tapetoretinal degeneration in an experimental animal more significance in relation to human pathology.

Although the retinal pathology in the rat strain studied in chapter 8 has been investigated extensively, the only certain conclusion that can be made is that there is a severe disturbance in the relationship between the photoreceptors and the pigment epithelium, resulting in an absence of phagocytotic activity in the pigment epithelium towards the rod outer segments.

Our findings concerning the development of the dystrophic rat retina include: a partial deficiency of rhodopsin regeneration capacity, a parallellism between rhodopsin content and rod outer segment retinoldehydrogenase activity, and an increased pigment epithelial retinoldehydrogenase activity, which parallels the amount of rod outer segment material outside the pigment epithelial cell. Large differences exist between animals kept in continual darkness and animals reared in cyclic light-dark conditions concerning the rhodopsin content. Such differences do not exist in the deficiency in rhodopsin regeneration capacity and the parallelism between rhodopsin content and enzyme activities. A problem in this type of experiments is the uncertainty whether a deviation represents the primary lesion or constitutes only a secondary effect. The differences in retinoldehydrogenase activities are probably secondary changes. In any case, a deficiency of these enzymes during the development of the rat can be excluded. The deficiency in rhodopsin regenerability in dystrophic animals may also be secondary to the anatomical changes, but since the regeneration process has not yet been elucidated, one can only guess at the defect underlying the disturbance in regenerability.

Further studies of rhodopsin regeneration in these animals are indicated because they provide an unique model system in which the retina and the pigment epithelium are gradually driven apart so that stages intermediate between a normal retina-pigment epithelium complex and complete isolation of the retina can be studied.

Although in these dystrophic animals no defect in retinoldehydrogenase activities is observed, the possibility exists that in some other

retinal disease a deficiency in ocular retinoldehydrogenases may exist. A deficiency of one of these enzymes would result in disease, because no other metabolic pathways for vitamin A present in the eye are known which can replace their function.

A claim that a general Na-K-ATPase deficiency in the dystrophic rat retina may be the primary cause of the retinal degeneration (Bonavita et al., 1966, 1967) is contradicted by our experiments. It is pointed out that a localized enzyme deficiency in the rod photoreceptor outer segments of the dystrophic animals can only be excluded by very sophisticated ultramicro techniques. The connection between such a localised enzyme deficiency and the anatomical changes however is not comprehensible.

The results of these experiments have also been published elsewhere (F.J.M. Daemen, J.J.H.H.M. de Pont, F. Lion and S.L. Bonting, 1970).

A comparison of the fatty acid distribution in retinas of normal rats and of dystrophic rat retinas has also been made. No gross differences are found, although minor differences cannot be excluded. Our tentative conclusion is that no defects in fatty acid synthesis or in protecting mechanisms against oxidation of fatty acids are present in rats with hereditary retinal dystrophy.

Het vitamine A speelt een belangrijke rol in de visuele excitatie van de lichtgevoelige receptoren in de retina, de eerste stap in een keten die leidt tot de visuele waarneming. Het vitamine A is, als essentieel bestanddeel van de visuele pigmenten in de retina, intensief betrokken bij de lichtgevoelige en metabole processen van de receptoren.

In dit proefschrift is getracht een aantal processen met betrekking tot de functie en het metabolisme van het vitamine A en het staafjespigment rhodopsine nader te onderzoeken. Daar deze processen uitsluitend in de retina plaatsvinden, kan een dergelijk onderzoek bijdragen tot een beter begrip van bepaalde erfelijke retinadegeneraties en van bijwerkingen van sommige geneesmiddelen op de retina.

In twee inleidende hoofdstukken wordt ingegaan op de anatomie en de fysiologie van de fotoreceptoren, in het bijzonder de staafjes van de retina. Een derde inleidend hoofdstuk behandelt enkele bij de mens voorkomende erfelijke degeneraties van de receptoren in de retina, met name retinitis pigmentosa, en geeft een overzicht over de klinische en experimentele onderzoekingen welke met betrekking tot dergelijke erfelijke degeneraties van het netvlies zijn verricht.

In het vierde hoofdstuk wordt aan de hand van een experimenteel model systeem, de geisoleerde retina van de albino rat, nagegaan, welke factoren een rol kunnen spelen in de regeneratie van het door licht gebleekte rhodopsine. De experimentele resultaten van Cone en Brown (1969) die onder speciale voorwaarden een spontane regeneratie van rhodopsine zagen, hebben wij niet kunnen bevestigen. In dit experimenteel model konden wij geen antwoord geven op de controverse in de literatuur, of aanwezigheid van het pigment epitheel al of niet noodzakelijk is voor het rhodopsine regeneratie proces.

In het vijfde tot en met het zevende hoofdstuk zijn experimenten bij retinas van runderen en ratten vermeld, welke betrekking hebben op enzymen die de omzetting bewerkstelligen tussen het vitamine A aldehyde en het vitamina A alcohol, de retinoldehydrogenases. De retinoldehydrogenase gelocaliseerd in de membranen van het staafjes-buitensegment blijkt uitsluitend de *all-trans* isomeer van het vitamine A als substraat te benutten. Op basis van deze gegevens wordt een vereenvoudigde rhodop-

sine-regeneratie-cyclus opgesteld. Het pigment epitheel blijkt eveneens een retinoldehydrogenase te bevatten, welke nog niet eerder is beschreven. Dit enzym heeft een duidelijke substraatvoorkeur voor het *11-cis* isomeer van vitamine A en verschilt ook in andere kenmerken duidelijk van de retinoldehydrogenase welke aanwezig is in de staafjesbuitensegmenten. Mede op basis van de intracellulaire localisatie van dit enzym - in een microsomale fractie rijk aan lysosomale enzymen - lijkt deze retinoldehydrogenase een rol te spelen in de afbraak van rhodopsine in het pigment epitheel, als onderdeel van de ononderbroken vernieuwingscyclus van de staafjesbuitensegmenten.

Het achtste hoofdstuk bevat de experimentele resultaten van enkele onderzoeken naar het metabolisme van de retina in een rattenstam met een autosomaal recessief erfelijke retinadegeneratie. Hoewel er grote verschillen optreden in het verloop van de retinale degeneratie in afhankelijkheid van ontwikkeling in het licht of in het donker, is in beide groepen de rhodopsine regeneratie-capaciteit duidelijk verminderd. De retinoldehydrogenase activiteiten lopen in de ontwikkeling van deze rat parallel aan het rhodopsine gehalte van de retina. In het pigment epitheel blijkt het daar gelocaliseerde enzym zelfs verhoogd in activiteit.

Hoewel een tekort aan Na-K-ATPase activiteit als oorzaak van deze retinadegeneratie werd aangemerkt door Bonavita et al. (1966, 1967), vinden wij tijdens de ontwikkeling van deze rat geen verschil in Na-K-ATPase activiteit vergeleken met normale ratten.

Mogelijke afwijkingen in het vetzuurspectrum van retinas van deze rattenstam zijn door ons niet gevonden.

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C U R R I C U L U M V I T A E

Franciscus Lion werd op 12 november 1943 geboren te Arnhem.
Na het behalen van het eindexamen Gymnasium α aan het Stedelijk Gymnasium te Arnhem in juni 1963, voldeed hij gedurende 18 maanden aan zijn verplichtingen ingevolge de dienstplichtwet.
In 1965 werd de studie in de Geneeskunde aangevangen aan de Katholieke Universiteit te Nijmegen, hetgeen in 1968 resulteerde in het kandidaatsexamen en in 1970 in het doctoraalexamen geneeskunde.
Het artsexamen werd behaald in januari 1973.
Sinds het kandidaatsexamen verrichtte hij als student-assistent gedurende een aantal perioden werkzaamheden op het Biochemisch Laboratorium van de faculteit der Geneeskunde van deze universiteit en van februari 1973 tot mei 1974 was hij als wetenschappelijk medewerker verbonden aan dit laboratorium. De in deze perioden verrichte onderzoekingen, uitgevoerd onder leiding van dr. F.J.M. Daemen en prof. dr. S.L. Bonting, hebben geleid tot het tot stand komen van dit proefschrift.
Sedert 1 mei 1974 is hij als arts-assistent werkzaam op de afdeling Oogheelkunde van de Katholieke Universiteit te Nijmegen (Directie: prof. dr. J.E.A. van den Heuvel en prof. dr. A.F. Deutman).

Stellingen.

1. Regeneratie van rhodopsine in het donker in afwezigheid van pigmentepitheel is nog niet overtuigend aangetoond.
   Cone, R.A. and Brown, P.K. (1969) Nature 21, 818-820
   Amer, S. and Akhtar, M. (1973) Nature New Biol. 245, 221-223.

2. Het onderzoek van Reading en Sorsby naar de retinoldehydrogenase in de retina van een rattenstam met een erfelijke netvliesdegeneratie is op experimentele gronden aanvechtbaar.
   Reading, H.W. and Sorsby, A.(1966) Biochem. J. 99, 3c-5c.

3. Het verschillend hanteren van de term "retinitis pigmentosa" door biochemici en oogartsen leidt gemakkelijk tot verwarrende conclusies bij biochemisch onderzoek van patiënten met een tapetoretinale degeneratie.
   Rahi, A.H.S. (1972) Brit. J. Ophthal. 56, 647-651.
   Futterman, S., Swanson, D. and Kalina, E. (1974) Invest. Ophthal. 13, 798-801.

4. Bestudering van de "myeloid bodies" in het pigmentblad van de retina in sommige vertebraten geeft mogelijk een beter inzicht in het visuele receptormechanisme bij de vertebraten.
   Nguyen-Legros, J. (1975) J. Ultrastruct. Res. 53, 152-163

5. De tegenstrijdige resultaten bij het opwekken van uveitis en retinitis in proefdieren zijn zowel verklaarbaar door het optreden van cross-reacties als door cross-contaminatie.
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6. Het is onjuist bij het ontwikkelen van methodieken voor weefseldifferentiatie met ultrageluid de apparatuur te standaardiseren met behulp van aan de te onderzoeken patiënt ontleende referentiewaarden.

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7. De term "kegeldysfunctie" dient gereserveerd te worden voor aandoeningen, waarbij electroretinografisch de kegelfunctie aangetast blijkt te zijn. Voor het bijbehorend kleurzindefect dient de term "type I verworven dyschromatopsie" gebruikt te worden.

François, J., de Rouck, A., Verriest, G., De Laey, J.J., Cambie, E. (1974) Ophthalmologica 169, 255-284.

8. De verschijnselen bij prematurenmyopie, retrolentale fibroplasie en het syndroom van Wagner doen een overeenkomstige pathogenese van deze aandoeningen vermoeden.

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9. Het retinale vitamine A gebruik is niet gecorreleerd aan de kijkdichtheidscijfers van de Duitse televisie.

F. Lion. 9 april 1976.